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# Characterization of Host Immune Responses to *Eimeria adenoeides* Infection in Turkey Poults

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Characterization of Host Immune Responses to *Eimeria adenoeides*  
Infection in Turkey Poults

Characterization of Host Immune Responses to *Eimeria adenoeides*  
Infection in Turkey Poults

A dissertation submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy in Poultry Science

by

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Sri Venkateswara Veterinary University  
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August 2013  
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This dissertation is approved for recommendation to the Graduate Council.

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## ABSTRACT

Coccidiosis is a common enteric disease of turkeys that is caused by protozoan parasites belonging to the genus *Eimeria*. There are about seven species of *Eimeria* that affect turkeys and of these *E. adenoeides* is the most pathogenic and commonly recognized. Infection with *Eimeria* is known to induce a long lasting protective immunity in chickens, but nothing is known regarding the acquisition of immunity to *Eimeria* in turkeys. The experiments reported here were aimed at investigating the biological and cellular immune response to *E. adenoeides* in turkey poults under different conditions of exposure. In experiment 1 (Chapter II), 20 day old poults were infected with  $12.5 \times 10^3$  oocysts and shown to develop immunity when challenged with a high dose of oocysts at 34 days of age, as judged by weight gain and oocyst production. Changes in peripheral blood leukocytes following the primary exposure were investigated, and an increase in the number of total lymphocytes, monocytes, and subsets of lymphocytes ( $CD4^+$  and  $CD8^+$ ) was shown in infected poults compared to uninfected controls.

In experiment 2 (Chapter III), local immune activities occurring in the ceca in response to infection were investigated. An increase in leukocyte infiltration, percent area occupied by  $CD4^+$  and  $CD8^+$  lymphocytes, and relative expression of cytokines (CXCLi2, IL1 $\beta$ , IFN $\gamma$ , IL10, and IL13) was observed in the ceca following infection. In experiment 3 (Chapter IV), we attempted to model the field situation by investigating the acquisition of immunity under conditions poults could encounter following placement upon built-up litter. Results indicated that they had acquired partial protection judged by weight gain and mortality by day 12 and 18 following challenge. The leukocyte infiltration score, percent area occupied by  $CD4^+$  and  $CD8^+$  lymphocytes, and expression of the cytokines (CXCLi2, IL1 $\beta$ , IFN $\gamma$ , IL2, IL10, IL12b, IL13, and

IL18) in the ceca significantly increased following infection. The changes in the ceca of *E. adenoeides* infected poult, in terms of infiltration of leukocytes, the area occupied by various subsets of lymphocytes, and the relative expression of chemokines and cytokines characterize the development of innate and adaptive immune activities.

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## **DEDICATION**

I dedicate this dissertation to my  
mother Ms. Ratna Kumari Gadde,  
father Mr. Manmadha Rao Gadde,  
husband Mr. Thilakar Rathinam,  
and  
my daughter Tvisha Rathinam



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### **CHAPTER II: Published**

Gadde, U., H. D. Chapman, T. Rathinam, and G. F. Erf. 2009. Acquisition of Immunity to the Protozoan Parasite *Eimeria adenoeides* in Turkey Poults and the Peripheral Blood Leukocyte Response to a Primary Infection. Poult. Sci. 88:2346-2352.

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## INTRODUCTION

Coccidiosis is an intestinal disease of turkeys, characterized by reduced feed intake, decreased weight gain, poor feed conversion, and high mortality in young poults. It causes great economic losses and costs billions of dollars to the poultry industry. One of the most common species in the turkey is *Eimeria adenoeides* and oocysts of this species, the transmission stage of the life cycle, were recently identified in almost all litter samples from commercial turkey farms submitted to our laboratory over a period of five years for drug resistance evaluation. Infection with *Eimeria* is known to induce a long lasting protective immunity in chickens, and extensive research has been carried out to determine the role of cells of the immune system in the development of immunity. Nothing is known regarding the acquisition of immunity to *Eimeria* in turkeys or the possible mechanisms involved.

The objectives of the study were to investigate the development of immunity to *E. adenoeides* in turkey poults and quantify the changes in numbers of leukocytes and expression of chemokines and cytokines in response to infection. The dissertation is divided into four chapters including the literature review. Chapter 2 submits to the characterization of immunity in 20 day old turkey poults and changes occurring in peripheral blood in response to infection. Chapter 3 includes the investigation of cellular, chemokine and cytokine changes occurring in ceca of 20 day old turkey poults in response to infection. Chapter 4 includes the investigation of development of immunity in newly hatched turkey poults to a series of doses of *E. adenoeides* infection that stimulates field exposure.

### **Chapter 1:** Literature Review

**Chapter 2:** Acquisition of Immunity to the Protozoan Parasite *Eimeria adenoeides* in Turkey Poults and the Peripheral Blood Leukocyte Response to a Primary Infection (Poult. Sci. 88:2346-2352, 2009).

**Chapter 3:** Cellular Immune Responses, Chemokine, and Cytokine Profiles in Turkey Poults following Infection with the Intestinal Parasite *Eimeria adenoeides* (Poult. Sci. 90:2243-2250, 2011).

**Chapter 4:** Acquisition of Immunity to the Protozoan Parasite *Eimeria adenoeides* in Turkey Poults and Cellular Responses to Infection (Submitted to Poult. Sci.).



**CHAPTER I**  
**REVIEW OF LITERATURE**

# COCCIDIOSIS

## *Introduction*

Coccidiosis is one of the commonest forms of enteropathy in poultry. It is a general term applied to infection with one or more species of protozoan parasites belonging to various taxonomic groups of the Class Sporozoa that includes *Eimeria*, *Toxoplasma*, *Sarcocystis*, and others. Coccidiosis caused by *Eimerian* parasites is primarily a disease of the intestine, is ubiquitous in nature, and has a distribution only limited by the availability of hosts and environmental conditions. Coccidiosis is characterized by reduced feed intake, retarded growth, poor feed conversion, morbidity, and occasionally mortality, all of which contribute to major economic losses in livestock production.

In the turkey industry, the introduction of intensive methods of rearing that involve raising large numbers of poults under one roof at high stocking densities has increased the occurrence of clinical coccidiosis (Clarkson and Gentles, 1958; Anderson et al., 1976; Joyner, 1978). Though mortality can be severe, losses caused by morbidity are of greater economic importance (Edgar and Bond, 1960; Bond, 1966). Worldwide, the annual cost of coccidiosis in chickens was estimated to be more than \$3 billion (Williams, 1999); although the economic cost of coccidiosis in turkeys has not been determined, it is likely that this is also substantial (Chapman, 2008). A major portion of the losses due to coccidiosis involve the recurring expenditure of medication for the prevention and treatment of the disease, and the high cost of vaccination.

Prolific use of drugs has resulted in the widespread development of drug resistant-strains (Chapman, 1997) and there is, therefore, an increasing need for the development of alternative,

efficacious measures of disease prevention, such as the development of recombinant vaccines. However, this will require a much better understanding of host-parasite interactions than we have at present and a thorough knowledge of the immune responses in the host that occur in response to infection.

### ***History of Eimeria***

Coccidia are microscopic, one-celled, protozoan parasites. The first protozoan observed was probably an oocyst of a species we now know as *Eimeria stiedai*, which was found in the gall bladder of rabbits by Antony Van Leeuwenhoek in 1674. More than 200 years later, Stieda (1865) described oocysts obtained from the liver of rabbits as early developmental stages of an unknown parasite and reported that they “segmented into separate bodies” when placed in water. Further developments occurred in 1879, when Leuckart introduced the generic name “*Coccidium*”, and placed this genus in the protozoan class Sporozoa. In 1896, Labbé placed oocysts from the mouse in the genus “*Coccidium*” and endogenous schizogony stages of the life cycle in the genus “*Eimeria*”. It was not until the description of the complete life cycle of *E. schubergi* from a centipede by Schaudinn (1900), that it was realized that oocysts and endogenous stages were in fact from the same organism and thus belonged in the same genus.

In 1910, Fantham provided the first detailed description of the life cycle of *Eimeria* from a bird (the Red Grouse *Lagopus lagopus*), Johnson (1923; 1923/1924) was the first to suggest the occurrence of more than one species of *Eimeria* in chickens and also introduced many important concepts such as host specificity and acquisition of immunity. The paper by Tyzzer (1929), now considered the most significant study concerned with *Eimeria* that has ever been published, gave

detailed descriptions of the life cycles of three chicken *Eimeria* sp. He also briefly described three new species in turkeys which he named *E. meleagridis*, *E. dispersa*, and *E. meleagrimitis*.

### ***Life cycle***

*Eimeria* are obligate intracellular parasites with complex life cycles involving both exogenous stages, such as the motile sporozoites and merozoites, and endogenous intracellular stages including schizonts and gamonts. The life cycle can be described as monoxenous, as development of the asexual schizonts and the sexual gamonts occur in the intestine of a single host. Transmission occurs by the oro-fecal route and the transmission stage is known as the oocyst.

The life cycle of *Eimeria* can be subdivided into three phases: sporogony, merogony, and gametogony. Sporogony occurs outside the host whereas merogony and gametogony are intracellular. Oocysts that pass in the feces are non-infectious when excreted and contain a single, undifferentiated zygote surrounded by a protective wall (unsporulated). Sporogony involves the formation of eight sporozoites within the oocyst and is an aerobic process that requires the presence of oxygen, adequate humidity, and optimal temperature. Nuclear divisions in sporogony involve meiosis, in which the zygote divides to form four haploid sporoblasts, followed by a mitotic division within each sporoblast to form two sporozoites. Once sporozoites are formed, a protective wall forms around each sporoblast, which is then known as a sporocyst. Thus the sporulated oocyst contains four sporocysts each with two sporozoites. Apart from the zygote, all subsequent stages of the life cycle are haploid (Canning and Anwar, 1968; Shirley and Millard, 1976).

Infection is established by the ingestion of sporulated oocysts by the host. In the intestine, oocysts are subjected to mechanical grinding and pressure by contraction of the smooth musculature of the gizzard and this results in breakage of the oocyst wall and release of the sporocysts (Doran and Farr, 1962). In the duodenum, sporocysts are exposed to bile salts, trypsin, and other proteases such as chymotrypsin, which cause the dissolution of a protein plug at the apex of each sporocyst and the release of sporozoites (Wang and Stotish, 1975; Chapman, 1978). Sporozoites invade epithelial cells at the tips of the villi in the species-specific region of the gut. Once inside epithelial cells, the sporozoites assume a rounded shape, and various subcellular organelles such as the apical complex that includes rhoptries, paired organelles, and dense bodies are lost. This stage is referred to as an immature schizont. The schizont undergoes repeated mitotic division and the newly formed nuclei migrate to the cell periphery where the cytoplasm invaginates around each nucleus to form stages known as merozoites. Once invagination is complete the mature schizont ruptures releasing large numbers of merozoites into the lumen of the intestine. These invade new epithelial cells, and undergo one or more cycles of schizogony before the onset of the sexual phase of the life cycle (gametogony). Gametogony involves the formation of two sexually differentiated stages, the macrogamont and the microgamont. The macrogamont develops into a uninucleate macrogamete and the microgamont divides to form numerous microgametes. Microgametes are motile, flagellated forms that fertilize the macrogamete and this results in the formation of a zygote. The zygote then secretes a double layered wall around itself to form the oocyst. Unsporulated oocysts are released into the intestinal lumen and discharged with feces.

### ***Host specificity***

*Eimeria* species are known to exhibit a marked degree of host specificity (Becker, 1948); for example, species that infect the chicken are not infective to turkeys and vice versa. Several experiments were conducted to test for the cross-infectivity of *Eimeria* species in both these hosts. Thus Tyzzer (1929) was unable to infect chickens with *E. meleagridis* from turkeys and turkeys with *E. acervulina* from chickens. Patterson (1933) failed to infect turkeys with *E. tenella* from chickens. Several unsuccessful attempts have been made to infect chickens, pheasants, guinea-fowls, pheasants, and Japanese quail with *E. adenoeides* of turkeys (Moore and Brown, 1951).

### ***Diagnosis***

Diagnosis of coccidiosis in chickens is based upon the characteristic pathological signs presented by each species and the presence of visible lesions in the intestinal tract. In the case of turkey *Eimeria*, this is difficult because readily identifiable lesions are not present. Demonstration of developmental stages (schizonts, gamonts) and large numbers of immature oocysts in intestinal scrapings are aids to diagnosis. Differential diagnosis between various species depends on the morphological characteristics of the oocysts, pathogenicity, and location of lesions and developmental stages within the host (Joyner, 1978). Several other criteria, such as the prepatent period, gross and microscopic lesions, and the absence of cross-protection between species should also be considered (Reid, 1972).

### ***Chemoprophylaxis***

In-feed medication with anticoccidial drugs is the preferred method of control as it is labor saving, cost effective, and enables intensive rearing of large numbers of birds commercially. The main aim of using anticoccidial drugs prophylactically is to prevent the birds from developing the acute form of the disease (Williams, 1969), a concept first introduced for the control of coccidiosis in chickens by Grumbles et al. (1948). The same concept has been adopted in turkey farming and usually poults are given anticoccidials in the feed from placement upon litter until 14 weeks of age.

Many factors are important in the selection of appropriate anticoccidial drugs for prevention of coccidiosis, such as efficacy, toxicity, withdrawal period, and effects upon the development of immunity (Mathis, 1993). Disadvantages of in-feed medication are cost, potential toxicity and development of drug resistance (Rose, 1976; Shirley, 1989). Studies were conducted to evaluate the sensitivity of field isolates of turkey *Eimeria* to various anticoccidial drugs and development of resistance to commonly used anticoccidials was reported (Chapman and Rathinam, 2007; Rathinam and Chapman, 2009).

### ***Immunoprophylaxis***

Vaccination is generally achieved by infection of birds with a small number of live oocysts of important species of *Eimeria*. Immunity then develops through repeated exposure of the poults to oocysts as a result of recycling of vaccinal parasites. Various methods have been employed for the administration of vaccines including eye-spray, application on the feed, and

administration in water. Recent techniques include the use of cabinets in the hatchery in which vaccine is mechanically sprayed directly onto the birds, and provision of vaccine to newly hatched chicks in an edible gel (Dalloul and Lillehoj, 2005; Lee and Soares, 2005).

Interestingly, despite their commercial availability, there are very few published studies that demonstrate the efficaciousness of coccidiosis vaccines. Use of live vaccines comprising drug-sensitive strains may result in the replacement of drug resistant strains in the field thereby alleviating the problem of drug resistance. Various schemes involving alternation of chemotherapy with vaccination have been proposed based upon this concept (Chapman, 1997), and these have been adopted by the poultry industry in so-called “rotation” programs. A concern exists that live vaccines may risk the introduction of species of *Eimeria* into farms that were not previously present. Live vaccines may under some circumstances cause vaccine-induced pathogenesis.



## IMMUNE RESPONSES

### *Avian immune system*

The avian immune system is highly complex and comprises two major components, the innate and the adaptive immune system (Lillehoj and Trout, 1993).

***Innate immunity.*** Innate immunity is inherently present and initiated upon the onset of infection. Its major elements include physical barriers such as the skin and mucus membranes, soluble factors such as complement, lysozyme, and acute phase proteins, and cells of the immune system including natural killer cells (**NK**), macrophages, and granulocytes.

NK cells are non-lymphoid, heterogeneous, and non-phagocytic, and can also be described as non-B, non-T, and non-macrophage mononuclear cells that possess cytotoxic activity against infected target cells (Lillehoj and Trout, 1994, 1996; Lillehoj and Lillehoj, 2000; Dalloul and Lillehoj, 2006). NK cells do not exhibit memory, lack MHC restriction, typically resemble large granulated lymphocytes, and are phenotypically defined as CD8<sup>+</sup> cells that lack expression of any characteristic T-lymphocyte markers (Rose, 1996; Lillehoj, 1998; Lillehoj and Lillehoj, 2000). They have been identified in various tissues in chickens including the spleen, peripheral blood, thymus, bursa, and the intestine (Schat et al, 1986; Chai and Lillehoj, 1988; Lillehoj and Chai, 1988; Lillehoj and Cheung, 1992). According to Gobel et al (2001), about 30% of CD8<sup>+</sup> intestinal intraepithelial lymphocytes (**IELs**) are NK cells. NK cells are known to play an important role in innate immune responses to parasitic infections and Lillehoj (1989) has demonstrated increased NK cell activity in the spleen and intestinal epithelium in response to *Eimeria* infection in chickens.

Macrophages are mononuclear phagocytic cells that are seen in various primary and secondary lymphoid tissues and play a significant role as scavengers in both innate and adaptive immunity (van Furth et al., 1972). Their major function in innate immunity is phagocytosis and they are also known to mediate inflammatory responses through production of various chemokines (Jeurissen et al., 1994; Abbas et al., 2010). They can act as antigen presenting cells in addition to dendritic cells and B lymphocytes. Their role in *Eimeria* infections in chickens is well established as they interact with the parasite during passage through intestinal mucosa (Vervelde et al., 1996; Hériveau et al., 2000). Dimier et al. (1998) studied the protective role of macrophages in inhibiting the development of sporozoites of *Eimeria* in cell cultures through production of cytokines. Dalloul and co-workers (2007) reported that in vitro, macrophages produce chemokines upon stimulation with sporozoites of *Eimeria*. Macrophages, in addition to T-lymphocytes, are also reported to be involved in the rejection of heterologous species of *Eimeria* from nonspecific hosts (Kogut et al., 1984).

***Adaptive immunity.*** Adaptive immunity involves the generation of specific, antigen-directed effector mechanisms and requires some time to develop after infection. A characteristic feature of adaptive immunity is the development of memory and an increased response to reinfection with the same pathogen. Major components include B- and T-lymphocytes (Lillehoj and Trout, 1993; Abbas et al., 2010). Lymphocytes develop and attain functional maturity in primary lymphoid organs which in birds are the thymus and bursa of Fabricius. Upon encounter with antigens, they differentiate into effector cells in secondary lymphoid organs, the spleen and mucosal associated lymphoid tissues. Depending on the type of antigen, two types of adaptive immunity can occur, antibody-mediated (humoral) immunity and cell-mediated immunity (**CMI**). Humoral immunity acts through production of antibodies which mediate immune

responses against extracellular pathogens through activation of complement, facilitation of phagocytosis by opsonization, agglutination or precipitation of antigen, neutralization, and antibody dependent cellular cytotoxicity. CMI is directed towards intracellular pathogens and the immune response is mediated by activation of macrophages and cytotoxic T-cells (Abbas et al., 2010).

Avian B-lymphocytes develop in the bursa of Fabricius, a primary lymphoid organ located near the cloaca, and are the effector cells of humoral immunity. They play an important role in the production of antigen specific antibodies. Activation of naive B-cells requires interaction with helper T-cells and once activated B-cells differentiate into plasma cells that produce antibodies. The primary antibody response involves production of the IgM isotype, which, following secondary exposure to antigen, changes to IgG or IgA. In avian species, three classes of antibodies have been described-IgM, IgA and IgY. IgY is considered the avian orthologue of mammalian IgG (Leslie and Clem, 1969) though the cDNA encoding IgY heavy chain is similar to IgE (Parvari et al., 1988).

T-cells form the antigen specific component of CMI (Chen et al., 1991). T-cell maturation occurs in the thymus, and mature T-lymphocytes leave to populate the secondary lymphoid organs and also circulate in peripheral blood and the lymphatic system. There are two major subpopulations of mature T-cells based upon their function- T helper lymphocytes (**Th**) and cytotoxic T lymphocytes (**Tc**). Th cells mediate antibody synthesis and delayed type hypersensitivity whereas Tc cells mediate cytotoxicity (Lillehoj and Trout, 1994; Abbas et al., 2010). Th cells respond to exogenous protein antigens when presented by the MHC class II receptors on antigen presenting cells, whereas Tc cells respond to endogenous antigens in association with MHC class I. Th and Tc cells can be distinguished phenotypically by their

expression of cell surface molecules, whose identification was made possible by the use of various monoclonal antibodies (Chan et al., 1988; Lillehoj et al., 1988). Typically, Th cells exhibit CD4 receptors and Tc cells show CD8 receptors on their surface membrane, though the expression of these molecules is not absolutely indicative of function.

Two types of Th cells exist and are referred to as type 1 (**Th1**) and type 2 (**Th2**) (Arstila et al., 1994; Erf, 2004; Abbas et al., 2010). Th cells can also be distinguished into two subsets (Th1 and Th2) based on the cytokines produced and effector mechanisms initiated (Mosmann and Coffman, 1989). Th1 cells produce interleukin 2 (**IL2**), interferon gamma (**IFN $\gamma$** ), and tumor necrosis factor (**TNF $\alpha$** ) which direct the response towards CMI and initiate other effects including delayed hypersensitivity, and macrophage activation. Th2 cells secrete IL4, IL5, IL6, IL13, transforming growth factor (**TGF**)  $\beta$ , and IL10 which help initiate humoral responses including antibody production and isotype switching (Mosmann and Coffman, 1989; Ovington et al., 1995; Jankovic et al., 2001; Erf, 2004; Abbas et al., 2010). Thus, responses to infection are greatly dependent on the type of subset of Th cells activated, though the occurrence of exclusive Th1 or Th2 responses is rare. Usually, activation of both subsets occurs, but the abundance of certain Th cytokines determines the path of response either towards a humoral or CMI (Jankovic et al., 2001; Gause et al., 2003).

### ***Avian Gut Associated Lymphoid Tissue (GALT) and its role in Eimeria infections***

The gut is a major immune organ that is able to combat exposure to various pathogens (Rose, 1996). It is the largest lymphoid organ in the body in terms of the number of lymphoid cells (Kraehenbuhl and Neutra, 1992). As *Eimeria* sp. are intestinal parasites, GALT forms the first

line of defense against infection (Lillehoj and Lillehoj, 2000). GALT is comprised of lymphoid tissue that is diffusely distributed both in epithelium, and in lamina propria (Lillehoj and Trout, 1996; Rose, 1996). The lymphoid tissue is also associated with various other groups of cells like antigen presenting cells (macrophages, dendritic cells), inflammatory cells (heterophils, mast cells, and eosinophils), and NK cells (Rose, 1996; McDonald, 1999). In addition to the diffuse lymphoid tissues, avian GALT also includes organized lymphoid structures such as the bursa of Fabricius, cecal tonsils, and Peyer's patches. Cecal tonsils are discrete lymphoid nodules located at the ileocecal junction, containing lymphoid tissue with germinal centers (Befus et al., 1980; Glick et al., 1981; Lillehoj and Trout, 1996). Peyer's patches are nodules of lymphoid tissue in the submucosa with a specialized, morphologically distinct, lympho-epithelium, microfold cells, a B cell dependent sub epithelial zone, and a T cell dependent central zone (Befus et al., 1980).

The composition of leukocytes in the intestine of birds includes approximately 80% lymphocytes, 10-15% macrophages, approximately 5% mononuclear cells, and less than 1% polymorphonuclear leukocytes and plasma cells (Befus, 1980; Lillehoj and Trout, 1994). Lymphocytes in the gut mucosa include IEL and the functionally distinct lamina propria lymphocytes (**LPL**). IEL are a phenotypically and functionally discrete subset of lymphocytes distinct from T-cells that occur in circulation (Ovington et al., 1995; Rose, 1996). The majority of IEL are  $CD4^-CD8^+$ , but also include  $CD4^+CD8^-$ ,  $CD4^+CD8^+$ , and  $CD4^-CD8^-$  T-cells (Bucy et al., 1988; Chen et al., 1988; Lillehoj and Trout, 1994; Ovington et al., 1995). Most IEL express T cell receptor 1 (**TCR1**), although a small proportion expresses TCR2. LPL comprises both B- and T-cells organized in clusters; the majority of LPL B-cells are positive for IgM or IgA, the T-cells are  $TCR2^+$  and  $CD4^-$  (Rose, 1996).

The immune responses that occur in the GALT are highly coordinated and include lymphocyte stimulation, cytokine secretion and activation of resident cells such as macrophages (Yun et al., 2000a). Antigens recognized by antigen presenting cells (dendritic cells, macrophages) are carried to the inductive sites, primarily PP, where they are presented for antigen recognition and immune cell activation. The activated T- and B-cells migrate to the lamina propria, which is the effector site for the immune response (Lillehoj and Trout, 1996). Intestinal immune responses include a complex interplay of soluble factors, cytokines, leukocytes, epithelial cells, endothelial cells and other physiological factors of GALT (Lillehoj, 1998).

### ***Induction and expression of immunity***

*Eimeria* species are known to induce a state of long lasting protective immunity in the host in response to repeated infections although this may vary depending upon the particular species involved. Thus in chickens infection with just a few oocysts of *E. maxima* can elicit a strong immune response whereas *E. tenella* does not. The relative immunogenicity of turkey species is not known. Immunity can be described as resistance to reinfection as measured by reduction in lesions in the intestines caused by the parasite and reduced reproduction of the parasite in the host (Rose, 1987, 1996). Birds that are protected do not lose weight when challenged with large numbers of the parasite.

Immunity to coccidial infections involves both innate and acquired immunity, the former playing a major role in elimination of parasites during the early phase of the primary infection and the latter during secondary infections (Lillehoj and Trout, 1994). Innate immunity involves non-specific natural immunity that lacks memory and the adaptive immunity involves specific

response that increases with each exposure (Rose, 1996). The two systems interact with each other and the innate immunity acts to augment the specific immunity.

Immune responses to *Eimeria* sp. are complex owing to the presence of exogenous and endogenous developmental stages of the life cycle (Rose, 1987). The responses vary greatly according to the stage of the life cycle, antigen composition of various stages, and magnitude of the antigenic stimulus. The asexual schizonts are considered to be more immunogenic than the sexual gamonts (Rose, 1974). Upon infection with *Eimeria* sp., both humoral and cell mediated responses are seen in the host (Lillehoj and Trout, 1994, 1996).

***Humoral immune responses.*** *Eimeria* species are known to induce the production of antibodies both in circulation and in mucosal secretions in response to a primary infection (Rose et al., 1984; Lillehoj, 1987a; Nash and Speer, 1988; Lillehoj and Trout, 1996). The kinetics and isotypes of antibody production are similar in avian, murine and bovine hosts (Rose, 1996). Antibodies detected in serum include IgM, IgG, and IgA (Trees et al., 1985; Lillehoj and Ruff, 1986). Antibodies in mucosal secretions include secretory IgA and were detected in bile, intestinal mucus and gut washings (Rose et al., 1984; Lillehoj and Ruff, 1986). However, the titers in serum do not correlate with the level of protection observed (Lillehoj et al., 1989a).

The primary response to infection included the production of serum IgM antibodies, which in later stages were replaced by IgG. Secondary infections were dominated by IgG, and only traces of IgM were seen (Rose and Mockett, 1983; Rose et al., 1984, Trees et al., 1985; Mockett and Rose, 1986). Davis et al. (1978) first reported an increase in the number of IgA secreting cells in gut mucosa and increased secretion of secretory IgA in response to *Eimeria* infection. Traces of IgA were also detected in serum in response to infection with *Eimeria* (Trees et al., 1985). There have been several reports describing specific secretory IgA responses

in bile and the gut mucosa in *Eimeria* infections in chicken and rodents (Douglass and Speer, 1985; Lillehoj, 1987a, 1989; Mockett and Rose, 1986; Rose et al., 1984; Vervelde et al., 1996; Wiesner, 1979).

Though antibody production was demonstrated in *Eimeria* infections, their role in conferring protection against coccidiosis is debatable. This is evident by the fact that bursectomized chickens with defective B cell production developed complete resistance to challenge infections (Rose and Long, 1970; Giambrone et al., 1981; Lillehoj, 1987b). Hence, the role of humoral immunity in limiting the infection is considered of little significance. However, in vitro studies have shown that antibodies from immune sera cause an increase in phagocytosis of sporozoites and merozoites by macrophages (Onaga and Ishii, 1980; Bekhti and Pery, 1989). Protection was also conferred in vivo by passive transfer of serum from immune hosts, injection of monoclonal antibodies produced to parasite antigens, and also maternal transfer of antibodies via yolk, indicating a protective humoral response against the disease (Wallach et al., 1990, 1992; Larsen et al., 1991).

The mechanism of action by which antibodies confer protection in *Eimeria* infections is not clearly understood. They are most likely to be directed against the extracellular stages of the parasite, sporozoites and merozoites (Wakelin and Rose, 1990). Several theories were proposed suggesting that they prevent the entry of sporozoites into the gut epithelial cells at the surface of the lumen. However, penetration into epithelial cells occurs even before the generation of specific antibodies and this may reduce parasite invasion in some, but not all *Eimeria* species (Lillehoj and Trout, 1996). Thus, humoral immune responses occupy a minor role in protection against coccidiosis, but in conjunction with cell mediated responses function to augment the host protective immunity.



Very little research has been conducted on investigating the humoral immune responses to *Eimeria* infections in turkeys. Augustin and Ridges (1963) reported the presence of antibodies in serum capable of precipitating the metabolic products or inner contents of merozoites of *E. meleagridis*. However, protective role of these antibodies was not demonstrated.

***Cell-mediated immune responses.*** Cell-mediated immune responses are the major immune responses that confer resistance to *Eimeria* infections. The important role of T-lymphocytes was demonstrated by the use of immunosuppressive drugs that selectively act on immunocompetent T-cells, such as cyclosporine-A, corticosterone and dexamethasone (Lillehoj, 1987b; Graat et al., 1997). Hosts deficient in T cell function show increased susceptibility or fail to develop immunity when challenged providing evidence that these are the major cells involved in immunity (Mesfin and Bellamy, 1979; Rose and Hesketh, 1979). Additional evidence for the protective role of T-lymphocytes was provided by in vitro studies of inhibition of intracellular development of sporozoites in cell cultures by T-cells isolated from immune chickens (Miller et al., 1994).

CMI elicited by *Eimeria* infections include both specific and nonspecific components and involve soluble factors, NK cells, Th and Tc cells (Lillehoj and Trout, 1994). Adoptive transfer or in vivo depletion of T-cells have shown that CD4<sup>+</sup> cells were more effective than CD8<sup>+</sup> cells during primary infection, and CD8<sup>+</sup> cells were more effective during secondary infections (Rose et al., 1988; Rose et al., 1992; Trout and Lillehoj, 1996). Overall, CD8<sup>+</sup> T cells mediate host immunity to coccidia in chickens (Lillehoj, 1994).

*Eimeria* infections are known to induce marked changes in the composition of T-cells in response to infection. With the use of techniques like immunohistochemistry, flow cytometry, and production of monoclonal antibodies against T-cell surface antigens, T cells have been

characterized into subsets and the changes in their numbers following infection investigated. Increase in the numbers of T cells in the peripheral blood was reported in infection with *E. maxima* and *E. tenella* in chickens (Rose et al., 1979). Lillehoj and Bacon (1991) reported an increase in CD8<sup>+</sup> IEL in the duodenum in *E. acervulina* infection. In situ characterization of T-lymphocytes, by immunohistochemistry, in the intestines revealed increased numbers of CD4<sup>+</sup> and CD8<sup>+</sup> cells in chickens infected with *E. maxima* (Rothwell et al., 1995) or *E. tenella* (Vervelde et al., 1996). Bessay et al. (1996) reported an increase in the numbers of CD4<sup>+</sup> and CD8<sup>+</sup> IEL in the duodenum of *E. acervulina* infected chicken, and in the ceca of *E. tenella* infected chicken. Differences in the proportions of CD4<sup>+</sup> and CD8<sup>+</sup> IEL were shown in two different genetic lines of chicken in response to *Eimeria* infection (Lillehoj, 1994; Yun et al., 2000a).

Drake et al. (2001) investigated changes in peripheral blood leukocytes, and macrophage function during a primary infection with *E. adenoeides*. Circulating lymphocytes were shown to be reduced 4 days after infection, whereas heterophils and lymphocytes were elevated in concentration 11 days after infection. There were no changes in nitric oxide production or phagocytic activity of macrophages.

### ***Cytokines and chemokines in coccidiosis***

Cytokines are small structurally diverse proteins or peptides that are briefly secreted by cells of the immune system in response to microbes and antigens. Cytokines play an important role in activation and regulation of immune and inflammatory responses (Wigley and Kaiser, 2003; Abbas et al., 2010). Their actions are pleotropic (one cytokine acts on different cell types) and

redundant (multiple cytokines have same function). They may stimulate production of other cytokines (e.g., IL12 increases IFN $\gamma$  production), initiate actions by binding to cell surface receptors, and their cellular response involves changes in gene expression in target cells leading to activation or down regulation of cellular activity (Abbas et al., 2010). Fewer cytokines have been described in birds than in mammals. Based on their function, cytokines may be categorized as pro-inflammatory (IL1 $\beta$ , IL6, IL8), Th1 (IFN $\gamma$ , IL2, IL18), and Th2 (IL3, IL4, IL5, IL13) (Wigley and Kaiser, 2003).

Chemokines are a small group of structurally related cytokines that play a major role in attracting leukocytes. They are classified into two major subfamilies based on the arrangement of terminal cysteine residues-CXC chemokines and CC chemokines. CXC chemokines attract neutrophils, whereas CC chemokines attract monocytes, lymphocytes, and eosinophils (Wigley and Kaiser, 2003). Their major functions include migration of leukocytes to the site of injury, promotion of adhesion of leukocytes to the endothelium, hematopoiesis, angiogenesis, and growth regulation (Laing and Secombes, 2004; Abbas et al., 2010).

*Eimeria* infections are known to induce the production of various chemokines and cytokines in response to primary and secondary infections including IFN $\gamma$ , TNF $\alpha$ , TGF $\beta$ , IL1 $\beta$ , IL2, IL6, IL8, IL12, IL16, and IL17. Extensive research was conducted on the changes in cytokine secretion in chicken infected with *Eimeria* sp., but until this study no such work has been done in turkeys.

**TNF.** TNF is produced mainly by activated macrophages and, to some extent, by antigen-stimulated T cells, NK cells and mast cells (Qureshi, 1998; Abbas et al., 2010). The principal function of TNF involves recruitment of neutrophils and monocytes to site of infection. There have been several reports of production of TNF in sera of chickens in response to *Eimeria*

infections (Byrnes et al., 1993a; Zhang et al., 1995a, b; Smith and Ovington, 1996; Rautenschlein et al., 1999) but none in the turkey.

**TGF.** TGF is an important cytokine that plays a major role in mucosal repair mechanisms following injury. It is also an important regulator of inflammation and exhibits pro-inflammatory properties at low concentrations and anti-inflammatory properties at higher concentrations (Omer et al., 2000). TGF isoforms have both growth promoting and growth inhibitory properties, and the effect that dominates depends on the effector cells and the presence of other cytokines (Roberts et al., 1985). Expression of TGF $\beta$ 4 was found to be significantly elevated in intestinal IEL of chickens following infection with *Eimeria* sp. (Jakowlew et al., 1997; Choi et al., 1999; Hong et al., 2006 a, b).

**IFN $\gamma$ .** IFN $\gamma$  has a multitude of immunomodulatory effects on a wide variety of tissues and was shown to play an important role in conferring protection against *Eimeria* infections. Rose et al. (1989) showed that in primary infections with *E. vermiformis* in mice, depletion of IFN $\gamma$  using monoclonal antibodies resulted in enhancement of infection in naïve BALB/c mice, indicating its role in limiting primary infections. Recombinant IFN $\gamma$  produced by antigen-stimulated immune T-cells inhibited the invasion and development of *E. tenella* sporozoites in cultured cells (Kogut and Lange, 1989; Lillehoj et al., 1989b; Dimier et al., 1998; Lillehoj and Chai, 1998), and improved weight gain in *E. acervulina* infected chickens in vivo (Lowenthal et al. 1997). A difference in the production of IFN $\gamma$  in spleen cells was reported in chickens following a primary infection with *E. maxima* and *E. tenella* (Prowse and Pallister, 1989; Byrnes et al., 1993a). Recent reports have shown the changes in expression of IFN $\gamma$  gene in response to coccidiosis in chickens using quantitative PCR and gene expression profiling (Choi et al., 1999; Yun et al., 2000b; Laurent et al., 2001; Hong et al., 2006a, b; Cornelissen et al., 2009).

**IL1 $\beta$ .** IL1 $\beta$  is a pro-inflammatory cytokine and is produced by activated mononuclear phagocytes, neutrophils, epithelial, and endothelial cells (Abbas et al., 2010). It acts as a mediator of the host inflammatory response by stimulating fibroblasts and epithelial cells to secrete chemokines. The role of IL1 $\beta$  in *Eimeria* infections in chickens is well established and several reports describe its production in vitro (Byrnes et al., 1993b), and changes in gene expression in vivo (Laurent et al., 2001; Hong et al., 2006a, b)

**IL2.** IL2 functions as a growth, survival and differentiation factor for T lymphocytes and NK cells (Farner et al., 1997; Abbas et al., 2010), and is mainly produced by T-cells especially CD4<sup>+</sup> lymphocytes. A significant difference in the expression of IL2 mRNA was showed in *Eimeria* infections in chickens (Choi and Lillehoj 2000; Li et al., 2002; Hong et al., 2006a, b; Cornelissen et al., 2009). The response was augmented and occurred earlier in secondary infections compared to primary infections indicating the role of IL2 in anamnestic response to coccidiosis (Miyamoto et al., 2002). Recently, the protective role of IL2 in enhancing the effect of recombinant vaccines was also reported. Injection of IL2 with 3-1E coccidia gene increased the host response to recombinant vaccine (Lillehoj et al., 2000; Min et al., 2001).

**IL6.** Macrophages, endothelial cells, and T-cells are the principal sources of this cytokine. IL6 is responsible for the final maturation of B-cells into antibody-producing cells and their proliferation (Abbas et al., 2010). IL6 activity was reported in the serum of chicken infected with *E. tenella* indicating its role in acquired immunity (Lynagh et al., 2000). Expression of IL6 gene was significantly increased in IEL of chicken infected with *E. acervulina*, *E. maxima*, and *E. tenella* (Hong et al., 2006a, b).

**IL4.** IL4 is produced mainly by CD4<sup>+</sup> T-cells of the Th2 subtype, activated mast cells, and NK cells. IL4 stimulates the development of Th2 cells from naïve CD4<sup>+</sup> cells and also has a

role in their differentiation (Abbas et al., 2010). It is also the principal cytokine that regulates the maturation and differentiation of B-lymphocytes and synthesis of immunoglobulins (Howard et al., 1982). Together with IL10, IL4 inhibits Th1 response by down regulating the production of IL12 (Brown and Hural, 1997). IL4 expression was shown to be altered in IEL of chickens infected with *E. acervulina*, *E. tenella* or *E. maxima* (Hong et al., 2006a, b). Annamalai and Selvaraj (2012) investigated the protective effect of in ovo IL4 plasmid injection against coccidial infection in chickens.

**IL13.** IL13 is structurally and functionally homologous to IL4 and is produced by CD4<sup>+</sup> helper T cells of the Th2 subset, CD8<sup>+</sup> cells, NK cells, basophils, and eosinophils (Abbas et al., 2010). IL13 has several functions like induction of IgE class switching in B cells, promotion of fibrosis in tissue repair, and also a pro-inflammatory role. A decrease in expression of IL13 in IEL was observed in primary and secondary infections with *E. acervulina* or *E. tenella* and an increase in the expression was noted in infections with *E. maxima* in chickens (Hong et al., 2006a, b).

**Other cytokines.** Changes in several other cytokines were reported in *Eimeria* infection in chickens. IL18 mRNA was found to be elevated in intestines of chicken infected with low and high doses of *E. acervulina* or *E. maxima* (Hong et al., 2006a, b; Swinkels et al., 2006, 2007). IL12 was shown to be increased in IEL of chickens infected with *E. acervulina*, *E. tenella* or *E. maxima* (Hong et al., 2006a, b; Kim et al., 2008).

## COCCIDIOSIS IN TURKEYS

### *Introduction*

The presence of *Eimeria* in turkeys was first noted by Theobald Smith in 1895 who found oocysts in the intestines of a young poult (Becker, 1934). The first species of *Eimeria* to be described in turkey was *E. meleagridis* (Tyzzer, 1927). Tyzzer (1929) identified and described two other species, *E. dispersa* and *E. meleagrititis*. Twenty two years later, Hawkins (1950) identified another new species which he named *E. gallopavonis*. Moore & Brown (1951, 1952) isolated and identified two other species and named them *E. adenoeides* and *E. innocua* respectively. The seventh species, *E. subrotunda* was reported by Moore et al. (1954).

Of the seven species of *Eimeria*, *E. meleagrititis*, *E. adenoeides*, and *E. gallopavonis* are considered to be highly pathogenic. *E. dispersa* and *E. meleagridis* are considered relatively pathogenic whereas all the other species are believed to be non-pathogenic (Lund and Farr, 1965). With the exception of *E. meleagridis* which develops in the mid-intestine and ceca (Hawkins, 1952; Clarkson, 1959a; Matsler and Chapman, 2006), *Eimeria* species are highly site specific and localize in a particular region of the gut. *E. meleagrititis* develops in the upper jejunum and to a lesser extent the duodenum (Clarkson, 1959b) and *E. dispersa* parasitizes the duodenum and upper intestine (Doran, 1978; Long and Millard, 1979). *E. adenoeides* develops in the cecum, terminal inch of small intestine, and rectum (Clarkson, 1958) whereas *E. gallopavonis* parasitizes posterior ileum, cecum, rectum (Hawkins, 1952; Farr, 1964). *E. innocua*, and *E. subrotunda* develop in the duodenum, jejunum, and upper ileum (Moore and Brown, 1952; Moore et al., 1954).

### ***Eimeria adenoeides***

Oocysts of *E. adenoeides* were first identified and described in intestines and droppings of turkey poults from New York farms by Moore and Brown (1951). Oocysts of this species are ellipsoidal in shape and narrower at one end with a length ranging from 18.95 - 31.26  $\mu$  and a width from 12.60 – 20.94  $\mu$  (Moore and Brown, 1951; Clarkson and Gentles, 1958). Average size of the oocysts is 25.60 X 16.25  $\mu$  (Clarkson, 1958; Clarkson and Gentles, 1958; Joyner, 1973) with a highest shape index of 1.54 (Moore and Brown, 1951; Reid, 1972). The optimum temperature for sporulation is  $26 \pm 1^{\circ}\text{C}$  and is completed in 24 h (Clarkson, 1958). The sporulated oocysts show one to three refractile bodies at one end.

Clarkson (1958) provided a detailed description of the life cycle and pathogenicity of this species. He found that *E. adenoeides* develops in the lower ileum, ceca and rectum of the intestine of poults and undergoes two asexual generations of multiplication prior to gametogony. The minimum prepatent period (time interval between infection and the first appearance of oocysts in feces) for *E. adenoeides* was shown to be 112 h (Moore and Brown, 1951) and sometimes may be as long as 132 h (Clarkson, 1958).

### ***Clinical signs of E. adenoeides infection***

Poults infected with *E. adenoeides* show symptoms from the fourth day following infection. Signs are not pathognomonic but include refusal to feed, anorexia, listlessness, droopiness, ruffled feathers, and a posture in which birds stand with their head between the legs. The feces become watery and show large amounts of orange colored mucus and only a few specks of



blood. By the end of the sixth day, solid, cheesy casts filled with oocysts are passed out in the feces. In severe infection, death occurs from 5-7 days following infection. Mild infections are characterized by weight loss (Moore and Brown, 1951; Clarkson, 1958; Clarkson and Gentles, 1958; Edgar and Bond, 1960; Lund and Farr, 1965).

Severity of infection is proportional to the number of oocysts ingested and the signs vary accordingly (Clarkson, 1958). Hein (1969) described the symptoms of *E. adenoeides* infection with various levels of doses and also explained the correlation between time of death, dose and stage of life cycle. As the level of infection increases, mortality tends to occur early. At higher doses, merogony seems to be responsible for mortality and at lower doses, gametogony coincides with mortality.

### ***Pathology and lesions of E. adenoeides infection***

*E. adenoeides* develops in the epithelial cells of the villi in the lower small intestine, cecum and rectum. The distinguishing feature of *E. adenoeides*, from other species infecting these regions such as *E. gallopavonis* and *E. meleagridis* is that the parasite develops in the crypts of Lieberkühn in addition to the villi (Moore and Brown, 1951; Clarkson, 1956).

Macroscopically, the lower small intestine and ceca become swollen, congested, and edematous and show petechial hemorrhages visible from the mucosal surface. By the 5<sup>th</sup> day, the lumen and cecal orifices become filled with strands of orange-colored mucus and solid, yellow caseous material composed of cellular debris and gametocytes. At the end of 6<sup>th</sup> day, the lumen gets filled with creamy white plugs containing large numbers of oocysts which have a characteristic cottage cheese like consistency (Clarkson, 1958; Clarkson and Gentles, 1958;

Lund and Farr, 1965; Reid, 1972; Joyner, 1973). There are a few reports concerned with the histological structure of the intestinal mucosa following infection. Ruff et al. (1981) reported a significant decrease in the villar height and increase in the depth of crypts of Lieberkühn in ileum of poult infected with *E. adenoeides*. Bemrick and Hammer (1979) reported that changes in cecal mucosa in response to *E. adenoeides* infection were most severe in the proximal end of the cecum, and different from those caused in cecal coccidiosis caused by *E. tenella* in chickens. Microscopically, the most striking early response to infection seen is the infiltration with eosinophilic leukocytes (Clarkson, 1958). It extends through the entire intestine in early phases of infection, but later becomes concentrated in the lower small intestine, ceca and rectum (Reid, 1972). Mucosal changes include desquamation, edema and increased cellularity of submucosa. The epithelial cells become cubical, reduced in size, and shows developmental stages.

### ***Physiological effects of E. adenoeides infection***

The effect of *E. adenoeides* on several physiological and metabolic parameters has been investigated. These effects include malabsorption of glucose and methionine in the jejunum, (Ruff et al., 1981), a significant reduction in heart weight, heart rate, and lipid levels in the heart (Augustine et al., 1982), a significant reduction in total plasma carotenoids, and a mild to no change in plasma and liver retinol (Augustine and Ruff, 1983). Changes reported in blood include decreased osmotic fragility of RBC (Augustine and Witlock, 1984), a significant increase in total serum proteins (Augustine and Thomas, 1981), and  $\alpha_2$ ,  $\gamma$ -globulin levels (Augustine, 1985), a significant reduction in serum albumin levels (Augustine, 1985), and an increase in plasma epinephrine, norepinephrine and catecholamine levels (Augustine and Denbow, 1991).

Muscle and plasma 3-methyhistidine levels were reported to be significantly increased indicating an increased muscle breakdown (Fetterer and Augustine, 2001). Infection with *E. adenoeides* was also shown to cause a reduction in plumage iridescence in male wild turkeys (Hill et al., 2005).

### ***Justification of present study***

*Eimeria adenoeides* is a widespread parasite that is capable of causing significant pathology in turkeys. Increasing feed costs, inefficacy of vaccines, increasing incidence of drug-resistant strains demand the need for exploring new avenues for better management of coccidiosis (Dalloul and Lillehoj, 2006). Development of novel recombinant vaccines with specific parasite antigens/genes that induce parasite-specific immunity would be preferable. This requires a comprehensive understanding of the avian immune system and means to elicit effective immunity against coccidia. A thorough knowledge of the interplay between the host and the parasite, and the immune responses generated in response to infection is also needed for developing newer methods of control.

Immune responses to *Eimeria* infections are complex and involve interplay of various types of cells and soluble cytokines. Extensive research was conducted in chickens regarding the development of immunity and the underlying mechanisms involved. Cells and cytokines conferring protection were characterized in both primary and secondary infections to commonly occurring species of *Eimeria* in chicken. Little or no such research was done in turkeys to characterize the immune responses to coccidian parasites. Hence, studies directed to delineate the roles played by different facets of the immune system in conferring protection to coccidiosis in turkeys are absolutely important.

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## **CHAPTER II**

### **ACQUISITION OF IMMUNITY TO THE PROTOZOAN PARASITE *EIMERIA ADENOEIDES* IN TURKEY POULTS AND THE PERIPHERAL BLOOD LEUKOCYTE RESPONSE TO A PRIMARY INFECTION**

**Acquisition of Immunity to the Protozoan Parasite *Eimeria adenoeides* in Turkey Poult  
and the Peripheral Blood Leukocyte Response to a Primary Infection**

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## ABSTRACT

A primary infection of  $12.5 \times 10^3$  oocysts of *E. adenoeides*, given to 20 d old turkey poults, resulted in depression of weight gain, and the production of large numbers of oocysts in the feces, compared with uninfected controls. Poults were raised under conditions to prevent possible re-infection in order to determine the ability of the primary infection to confer protective immunity against a challenge infection of  $5 \times 10^4$  oocysts given at 34 d of age. Using weight gain and oocyst production following challenge as criteria for protection, the results indicated that immunity had developed. The concentration and proportions among white blood cell (WBC) populations in peripheral blood were determined at different times following the primary infection. The WBC concentration of infected poults was elevated on d 7 and 11, primarily due to elevated levels of lymphocytes and monocytes on d 7, and eosinophils on d 11. There were no differences in heterophil and basophil concentrations between infected and uninfected poults at any of the time points examined. With the exception of increased percentages of eosinophils on day 11, infection was not associated with alterations in the proportions among WBC populations. Comparison of CD4- and CD8-defined lymphocyte subpopulations in the blood of infected versus uninfected poults revealed higher concentrations of CD4+ lymphocytes on d 11, lower concentrations of CD8+ cells on d 4, and higher concentrations of CD8+ cells on d 11 of infection, as well as elevated ratios of CD4+- to CD8+- lymphocytes in infected birds on d 4 and 11. These alterations in WBC profiles following primary *E. adenoeides* infection in turkey poults suggest initiation of both innate and adaptive cellular immune activities designed to effectively cope with a parasitic, intracellular pathogen.

**Key words:** *Eimeria*, turkey, immunity, leukocyte, cell-population analysis

## INTRODUCTION

Coccidiosis is a widespread disease of the intestinal tract of turkeys caused by parasites of the protozoan genus *Eimeria*. One of the commonest species in the turkey is *E. adenoeides* that can cause reduced feed intake, decreased weight gain, poor feed conversion, and high mortality in young poults (Clarkson, 1958). Oocysts of *Eimeria*, the transmission stage of the life cycle, were recently identified in almost all litter samples from turkey farms submitted to our laboratory for drug resistance evaluation. The majority of these isolates contained *E. adenoeides* (Rathinam & Chapman, In Press). Infection with *Eimeria* is known to stimulate a protective immune response in chickens (e.g. Rose, 1996; Yun et al., 2000) but almost nothing is known concerning the development of immunity to those species that infect the turkey. In this study we investigate the acquisition of immunity to *E. adenoeides* in turkey poults and determine white blood cell population profiles throughout the course of a primary infection.

## MATERIALS AND METHODS

### *Animals and Husbandry*

Female poults (Nicholas breed) were obtained from a local hatchery and transferred to brooder cages in an isolation building at the University of Arkansas. They were reared at a stocking density of 257 cm<sup>2</sup> / poult, and a brooder temperature of 35-38°C. All poults were fed a

nutritionally adequate corn-soybean diet and had free access to water (NRC, 1994). Husbandry followed guidelines for the care and use of animals in agricultural research (Anon, 1999).

### ***Experimental Design***

The experiment comprised two phases as indicated in Table 1. In the first phase poult were randomly allocated to two treatments and given either a primary inoculum of oocysts (treatment 1) or not infected (treatment 2; uninfected controls). In the second phase, poult in each of these two treatments were either challenged with a high dose of oocysts (treatments 1A and 2A) or not infected (treatments 1B and 2B; unchallenged controls).

### ***Primary Infection***

At 17 d of age poult were separately identified with a unique wing band number and allocated to eight clean battery cages, 12 poult / cage, at a stocking density of 460cm<sup>2</sup> / poult. Two separate test rooms were utilized. Four cages in one room were used for poult to be given the primary inoculum of oocysts; four cages in the other room housed the uninfected controls. Two rooms were used to prevent the possibility of accidental transfer of oocysts from the room containing infected birds to that containing the uninfected controls. Three birds from each cage were randomly identified for subsequent removal of a sample of peripheral blood (12 poult / treatment). Three d after allocation to cages, at 20 d of age, all poult were weighed and either inoculated orally with  $12.5 \times 10^3$  oocysts of *E. adenoeides* (primary inoculum), or given a sham dose of water (uninfected controls). Commencing on d 25 (5 d after infection), poult in all

cages were transferred daily to an adjacent cage that had been thoroughly cleaned in order to prevent accidental re-infection. This was continued on a daily basis until d 33 (the day before the challenge phase of the study). At 26 d of age all poult were weighed and weight gain from d 0-6 post infection calculated. Feces from each cage were collected from d 5-6, 6-7, 7-8, 8-9, and 13-14 post infection, and the numbers of oocysts present determined by the following procedure. Feces were homogenized in 5L of water, a 1mL sample diluted with 49 mL of saturated salt solution, and the number of oocysts counted in a McMaster chamber. Results were expressed as the total number of oocysts produced / poult for the 24 h period of collection. On d 0, 4, 7, and 11 after the primary inoculum a blood sample was collected alternately from the left and right brachial vein of the 12 poult that had been pre-selected from the infected and uninfected groups respectively.

### ***Challenge Infection***

At 34 d of age poult from two of the cages that had received the primary inoculum and from two cages containing uninfected birds were weighed, allocated to clean cages, and challenged with  $5 \times 10^4$  oocysts (treatments 1A and 2A). Poult from the remaining cages were also weighed and allocated to new cages but were not challenged (treatments 1B and 2B; unchallenged controls). Six d later (40 d of age) poult were weighed and weight gain from d 0-6 post challenge determined as described above. Feces were collected from d 5-6, 6-7, and 7-8 after challenge and the numbers of oocysts produced determined.

### ***Parasite***

The isolate of *E. adenoeides* was obtained from a litter sample collected from a turkey farm in Kansas in 2006 and had been purified from 3 oocysts using the low gelling agarose method described by Shirley and Harvey (1996). Oocysts were less than one mo old when used in experiments.

### ***Blood Samples***

Heparinized blood samples (1 mL) collected on d 0, 4, 7, and 11 after the primary inoculum were used to determine the concentrations of total white blood cells (**WBC**,  $10^3 / \mu\text{L}$ ), red blood cells (**RBC**,  $10^6 / \mu\text{L}$ ) and thrombocytes ( $10^3 / \mu\text{L}$ ) utilizing an automated hematology analyzer calibrated for turkey blood (CELL-DYN 3500SL System; Abbott Diagnostics, Abbott, Abbott Park, IL). The samples were also used to measure hemoglobin concentration, pack cell volume (**PCV**), mean corpuscular volume (**MCV**), and mean corpuscular hemoglobin. The proportions of the various WBC populations were manually determined using Wright stained blood smears (Lucas and Jamroz, 1961; Wang et al., 2003). Blood smears were observed using a bright field microscope with oil immersion at 1,000X magnification. Approximately 300 leukocytes, specifically lymphocytes, heterophils, monocytes, eosinophils, and basophils were identified and counted based on morphological characteristics (Lucas and Jamroz, 1961). The proportion of a leukocyte population was expressed as the percentages of total leukocytes examined. The heterophil to lymphocyte ratio (H / L ratio) was calculated by dividing the number of heterophils by the number of lymphocytes identified on each blood smear. The concentration of individual

leukocyte populations was calculated using the total WBC concentration estimated by the automated hematology analysis and the percentages of individual leukocytes estimated by manual differential leukocyte counts.

### ***Peripheral Blood Mononuclear Cell Isolation***

Peripheral blood mononuclear cells (**PBMC**) were isolated from the remaining whole blood (0.6-0.7 mL) for immunofluorescence staining and flow cytometric cell population analysis. To isolate PBMC, the blood was diluted in room temperature Dulbecco's phosphate buffered saline (**PBS**; Sigma Chemical Company, St. Louis, MO) at a 2:1 ratio, respectively. The blood dilution was layered on Fico / Lite LymphoH 1.077 (Ficoll; Sigma) at a 1:1 ratio in 5 mL Falcon tubes (Becton Dickinson, Franklin Lakes, NJ). The suspension was centrifuged in a swing-bucket centrifuge (Jouan CR312, Thermo Fisher Scientific Inc., Waltham, MA) at 350 x g for 30 min at room temperature. The PBMC interface between the Ficoll and the plasma layers was collected and washed two times with PBS at 250 x g for 8 min at 4°C. CD4- and CD8-defined lymphocyte subsets in the PBMC suspension were identified using a direct fluorescence staining procedure (Erf et al., 1998). The antibodies employed were mouse anti-chicken CD4-FITC (clone CT-4) and mouse anti-chicken CD8-PE (clone 3-298) (Southern Biotechnology Associates Inc., Birmingham, AL) known to cross-react with turkey CD4 and CD8, respectively. Controls included FITC- and PE-labeled isotype control (mouse IgG1 with irrelevant specificity; Sigma) to test for non-specific binding of the specific antibodies (mouse IgG1) and to set the cut-off between fluorescence-positive versus fluorescence-negative PBMC for FL-1 (FITC) and FL-2 (PE), respectively. The percentages of CD4+ and CD8+ live cells in the PBMC population were



determined by flow cytometry using a Becton Dickinson FACSort equipped with a 488-nm argon laser (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cell population analysis was conducted using CellQuest software. The percentage of CD4+ and CD8+ lymphocytes in the live, small mononuclear cell population (lymphocytes and thrombocytes), determined by flow cytometry, was converted into concentrations based on concentration estimates of lymphocytes and thrombocytes in whole blood.

### ***Data Analysis***

For the primary inoculum, 4 replicates of 12 poult / cage were used for measurement of weight gain and oocyst production; for the challenge inoculum, 2 replicates of 9 poult / cage were used for measurement of these criteria. Analysis of WBC populations and other blood parameters was based upon 4 replicates of 3 poult / cage and 2 replicates of 3 poult / cage for the primary and challenge inocula respectively. Cage means served as the experimental unit for statistical analysis. Weight gain, the number and proportion of WBC populations and other blood parameters were analyzed by one-way analysis of variance using SAS software (SAS Institute, 2003). Means were separated and compared using Student-Newman-Keul Test. No oocysts were produced by uninfected controls in these experiments and therefore oocyst production data were not statistically analyzed.

## RESULTS

### *Weight Gain and Oocyst Production – Primary Infection*

Infected poult (treatment 1) showed a lower weight gain ( $P < 0.001$ ) than uninfected birds (treatment 2) following inoculation with  $12.5 \times 10^3$  oocysts (Table 2). Large numbers of oocysts were produced in the feces of infected poult from d 5-6 followed by a decline. Small numbers were still produced from d 13-14 after infection. No oocysts were produced by uninfected controls. No mortality occurred because of infection.

### *WBC – Primary Infection*

The concentration of WBC from infected poult was elevated on d 7 and 11 after infection ( $P < 0.05$ ) compared with uninfected controls (Table 3). No significant differences in the concentration of heterophils or basophils in the peripheral blood were observed (Table 4). The concentration of lymphocytes and monocytes was higher in infected birds on day 7 after infection, eosinophil concentration was elevated on d 11 ( $P < 0.05$ ). No significant differences in the proportions of lymphocytes, heterophils, monocytes, basophils (Table 4) or H / L ratio ratios were observed (data not shown). The proportion of eosinophils was elevated on d 11 ( $P < 0.05$ ).

### ***CD4+ and CD8+ Lymphocyte Concentrations and Ratios – Primary Infection***

CD4+ lymphocyte concentration of infected poult was greater than uninfected controls on d 11 after infection (Table 5). CD8+ lymphocyte concentration of infected poult was less than the controls on d 4 but higher on d 11 ( $P < 0.05$ ). The ratio of CD4+ to CD8+ cells was elevated in infected birds on d 4 and 11 ( $P < 0.05$ ).

### ***Other Blood Parameters – Primary Infection***

No significant differences were found in total RBC, PCV, MCV, hemoglobin concentration, mean corpuscular hemoglobin concentration, and thrombocyte concentrations in the blood of infected and uninfected poult at different d following the primary infection (data not presented).

### ***Weight Gain and Oocyst Production – Challenge Infection***

Results are presented in Table 6. The weight gain of challenged poult that had received a primary inoculum (treatment 1A) and unchallenged controls (treatments 1B, 2B) was greater ( $P < 0.005$ ) than that of challenged poult that had not been infected (treatment 2A). This indicates that, using weight gain as a criterion for protection, immunity had developed to *E. adenoides*. Large numbers of oocysts were produced in the feces of challenged poult that had not received the primary inoculum (treatment 2A). By contrast few oocysts were produced in challenged birds that had been infected (treatment 1A) and none were produced in unchallenged controls.

This indicates that, judged by the ability of the parasite to multiply in the host, immunity had developed following the primary infection.

## DISCUSSION

*E. adenoides* is one of the most pathogenic species of *Eimeria* that infect the turkey and has been shown to cause reduced weight gain during the acute phase of infection (e.g. Clarkson, 1958; Hein, 1969). In this study, doses of  $12.5 \times 10^3$  and  $5 \times 10^4$  oocysts caused weight depression but no mortality. Large numbers of oocysts were produced following the primary infection but none were found in the uninfected poults. Similarly no oocysts were found in uninfected poults during the challenge phase of the study. This indicates that the procedure to prevent accidental infection from occurring in uninfected birds was successful. Poults that had not received a primary inoculum produced large numbers of oocysts when challenged at 34 d of age indicating that they remained susceptible to infection.

An objective of this study was to investigate whether poults acquire immunity because of a primary infection. According to Augustine (1988), a single dose of  $1.5 \times 10^5$  oocysts, in which equal numbers of *E. adenoides* and *E. meleagrimitis* were given to 14 d old poults, afforded good protection, judged by weight gain, against challenge two wk later with  $3 \times 10^5$  oocysts of the mixed species. Since two species were included in the inoculum it is not possible to determine their separate contributions to the development of immunity. An important practical consideration in the design of experiments to investigate the effects of a single dose of oocysts is the prevention of accidental exposure resulting from oocysts passed in the feces (Rose, 1974).

This may occur even if birds are raised on wire floors. In the experiment undertaken here, accidental exposure was avoided by transferring birds to clean cages on a daily basis during the patent period of the primary infection. It is considered likely, therefore, that any effects observed can be attributed to the primary inoculum.

The results indicate that, judged by weight gain and oocyst production, poult had acquired immunity to *E. adenoeides* as a consequence of exposure to  $12.5 \times 10^3$  oocysts. A few oocysts were produced by challenged poult that had received the primary infection suggesting that inhibition of parasite development was not complete. According to Rose (1996), clinical immunity precedes complete anti-parasite immunity, which appears to be a more stringent criterion for evaluating the immune response.

In the chicken, a single infection of the intestinal parasite *E. maxima* resulted in immunity (judged by inhibition of parasite development) whereas multiple infections were necessary for the pathogenic cecal parasite *E. tenella* (Rose, 1974). *E. adenoeides* is highly pathogenic and, like *E. tenella*, develops predominantly in the ceca; multiple infections were not necessary, however, for protection to be acquired. According to Rose (1985), there is no correlation between immunizing ability of different species and pathogenicity to the host, or the area of the gut parasitized. The finding that protection against the cecal parasite *E. adenoeides* develops after a single dose of oocysts supports the conclusion that area of the gut parasitized may not be important in immunizing ability.

No differences were found in RBC, hemoglobin concentration, PCV, MCV, and thrombocyte numbers following the primary infection with  $12.5 \times 10^3$  oocysts. In another study, PCV and whole blood hemoglobin were significantly higher six d after infection of poult with *E. adenoeides* but the number of oocysts given ( $10^5$ ) was substantially higher than in this

experiment (Augustine and Witlock, 1984). Osmotic fragility of RBC was also decreased which was thought due to reduced water intake. Changes in blood cell values are less likely in turkeys infected with *Eimeria* species because, unlike species such as *E. tenella* and *E. necatrix* that infect the chicken, little overt hemorrhage is observed (Clarkson, 1958).

An increase in the concentration of WBC was found in infected poult on d 7 and 11 after the primary infection. Infection was not associated with differences in the concentration of heterophils or basophils, but lymphocyte and monocyte concentrations were elevated on d 7 after infection as was the concentration of eosinophil on d 11. With the exception of eosinophils on d 11, proportions of the other leukocytes did not differ between infected and uninfected poults. Additionally, infected poults had higher concentration of CD4<sup>+</sup> lymphocytes on d 11, lower concentrations of CD8<sup>+</sup> lymphocytes on d 4, and higher concentrations of CD8<sup>+</sup> lymphocytes on d 11. These shifts in lymphocyte subpopulations were also reflected in the elevated ratios of CD4<sup>+</sup> to CD8<sup>+</sup> lymphocytes on d 4 and 11.

There have been few investigations of WBC responses to infection with *Eimeria* in poultry. A biphasic increase was observed following a primary infection of 4 wk old chickens with  $15 \times 10^3$  oocysts of *E. maxima*, peak numbers occurring from d 2-6 and 9-17 d after infection (Rose et al., 1979). Differential counts indicated a biphasic increase in polymorphonuclear cells, lymphocytes, and large mononuclear cells but no change in eosinophil numbers was found. PBL responses have also been investigated in 3 wk old turkeys infected with  $5 \times 10^4$  oocysts of *E. adenoeides* (Kogut et al., 1984). They observed a significant decrease in WBC 2 h after infection and a slight increase at 24 h, however, no differences were found from 2-6 d post inoculation. They found no significant changes in polymorphonuclear cells or lymphocyte numbers although there was a sustained increase in large mononuclear cells.

The alterations in the concentrations of WBC populations observed here, during primary infection with *E. adenoeides* in 20 d old turkey poults, are supportive of initiation of cellular immune activities involving components of both innate and adaptive immunity. It has been well-established that *Eimeria* infection in chickens requires cell-mediated immunity for resolution and protection (Yun et al., 2000; Hong et al., 2006). The increase in peripheral blood lymphocytes and monocytes on d 7 together with the increased CD4<sup>+</sup> to CD8<sup>+</sup> lymphocyte ratios on day 4 and 11, and the increase in CD8<sup>+</sup> lymphocytes on d 11 collectively point towards initiation of cell-mediated immunity. While further studies are needed to examine events occurring in secondary lymphoid organs, such as the spleen and cecal tonsils, and at the site of *E. adenoeides* infection (ceca), the blood does reflect the mobilization and recruitment of cells in response to infection. Hence it is likely that cell-mediated immunity is responsible for the observed protection in the challenge study. The increased concentration and proportions of eosinophils in the blood on d 11 suggests recruitment and participation of this granulocyte in the effector phase of the immune response. As eosinophils are generally known to have specialized in the elimination of extracellular parasites, their recruitment in response to *Eimeria* infection is not surprising and is reflective of directed anti-parasite inflammatory immune activity (Abbas et al., 2007). Myeloid cell responses (eosinophils, basophils, mast cells), both intestinal and peripheral, have been reported to accompany the development of immunity to *Eimeria*, however, the essential, specific T cell function was found to be independent of myeloid responsiveness (Rose and Wakelin, 1990). Taken together, based on changes in WBC populations during the course of a primary infection with *E. adenoeides* in turkey poults, effector immune mechanisms designed to deal with different stages of the parasites have been initiated (extracellular stages: eosinophils; intracellular stages: CD8<sup>+</sup> lymphocytes and monocytes/macrophages). While emphasis is on

cellular immune activities, both CD4+ T helper cell type 1 (Th1) and CD4+ Th2 lymphocytes are likely involved in the coordination of these protective efforts, an observation also reported for *E. tenella* and *E. acervulina* infection in chickens (Hong et al., 2006). Further studies are needed to address the immune mechanisms underlying the observed protective immunity in poult exposed to a primary infection of *E. adenoeides*.

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**Table 1. Experimental design**

Primary infection <sup>1</sup>				Challenge infection <sup>2</sup>			
Treatment	Cage number	Number of birds <sup>3</sup>	Infected	Treatment	Cage number	Number of birds <sup>3</sup>	Infected
1	1	12	Yes	1A	1	9	Yes
1	2	12	Yes	1A	2	9	Yes
1	3	12	Yes	1B	3	9	No
1	4	12	Yes	1B	4	9	No
2	5	12	No	2A	5	9	Yes
2	6	12	No	2A	6	9	Yes
2	7	12	No	2B	7	9	No
2	8	12	No	2B	8	9	No

<sup>1</sup>Poults were inoculated at 20 d of age with  $12.5 \times 10^3$  oocysts.

<sup>2</sup>Poults were challenged at 34 d of age with  $5 \times 10^4$  oocysts.

<sup>3</sup>Blood samples were taken from the brachial vein of 3 poults from each cage at different times following infection.

**Table 2. Weight gain and oocyst production of 20 d old turkey poultts infected with 12.5 x 10<sup>3</sup> oocysts of *E. adenoeides***

Treatment number <sup>1</sup>	Primary infection	Weight gain (g) <sup>2</sup>	Oocysts/poult (x 10 <sup>6</sup> ) <sup>3</sup>				
			5-6	6-7	7-8	8-9	13-14
1	Yes	198 ± 5 <sup>b</sup>	104 ± 20	37 ± 3	14 ± 1	12 ± 1	2 ± 0
2	No	246 ± 7 <sup>a</sup>	0	0	0	0	0
P-value		0.001	Not done				

<sup>1</sup>Each treatment comprised four cages of 12 poultts.

<sup>2</sup>Mean weight gain ± SEM from d 0-6 after the primary infection. Values in a column with no common superscript differ significantly.

<sup>3</sup>Oocysts produced/poult ± SEM from d 5-6, 6-7, 7-8, 8-9, and 13-14 after the primary infection.

**Table 3. Total white blood cell (WBC) concentrations ( $\times 10^6/\text{mL}$ ) in the peripheral blood of turkeys at different times following infection with  $12.5 \times 10^3$  oocysts of *E. adenoeides* at 20 d of age.**

Day of infection	WBC (mean $\pm$ SEM) <sup>1</sup>		P - value
	Treatment <sup>2</sup>		
	Infected	Uninfected	
0	36.0 $\pm$ 6.4 <sup>3</sup>	26.2 $\pm$ 3.3	0.188
4	27.1 $\pm$ 4.2	35.1 $\pm$ 4.3	0.196
7	32.8 $\pm$ 3.2 <sup>a</sup>	23.0 $\pm$ 2.6 <sup>b</sup>	0.027
11	25.7 $\pm$ 4.7 <sup>a</sup>	14.3 $\pm$ 2.0 <sup>b</sup>	0.037

<sup>1</sup>Values in a row with no common superscript differ significantly ( $P \leq 0.05$ ).

<sup>2</sup>Each treatment comprised 3 poult from four cages.

<sup>3</sup>WBC concentration was determined using a CELL-DYN automated hematology analyzer calibrated for turkey blood.

**Table 4. Concentrations and proportions (%) among white blood cells in peripheral blood of turkeys at different times following infection with  $12.5 \times 10^3$  oocysts of *E. adenoeides* at 20 d of age.**

Day of infection	Treatment <sup>1</sup>	Lymphocyte	Heterophil	Monocyte	Eosinophil	Basophil
Concentration ( $\times 10^6$ mL of blood) <sup>2,3</sup>						
0	Infected	$15.4 \pm 3.8$	$16.5 \pm 2.5$	$1.9 \pm 0.4$	$0.9 \pm 0.2$	$1.2 \pm 0.2$
0	Uninfected	$11.1 \pm 2.0$	$11.4 \pm 1.4$	$1.1 \pm 0.2$	$1.2 \pm 0.3$	$1.4 \pm 0.3$
4	Infected	$8.5 \pm 2.1$	$15.2 \pm 2.5$	$1.3 \pm 0.2$	$1.0 \pm 0.1$	$1.2 \pm 0.2$
4	Uninfected	$12.4 \pm 2.2$	$17.7 \pm 1.8$	$1.8 \pm 0.4$	$1.5 \pm 0.4$	$1.6 \pm 0.3$
7	Infected	$18.4 \pm 3.3^a$	$10.7 \pm 1.7$	$1.5 \pm 0.2^a$	$0.8 \pm 0.1$	$1.5 \pm 0.2$
7	Uninfected	$10.0 \pm 1.0^b$	$9.9 \pm 1.8$	$0.9 \pm 0.1^b$	$0.9 \pm 0.1$	$1.3 \pm 0.2$
11	Infected	$11.2 \pm 2.4$	$10.5 \pm 2.8$	$1.0 \pm 0.2$	$1.5 \pm 0.3^a$	$1.5 \pm 0.4$
11	Uninfected	$7.4 \pm 0.9$	$4.7 \pm 3.0$	$0.6 \pm 0.2$	$0.5 \pm 0.4^b$	$1.0 \pm 0.7$
Proportions (% of leukocytes) <sup>2</sup>						
0	Infected	$40.8 \pm 2.1$	$47.4 \pm 2.5$	$5.2 \pm 0.4$	$2.7 \pm 0.4$	$4.0 \pm 0.5$
0	Uninfected	$41.4 \pm 3.9$	$44.8 \pm 4.0$	$4.3 \pm 0.3$	$4.5 \pm 0.7$	$5.2 \pm 0.6$
4	Infected	$31.8 \pm 4.2$	$54.8 \pm 3.0$	$5.1 \pm 3.0$	$4.0 \pm 0.6$	$4.4 \pm 0.5$
4	Uninfected	$34.1 \pm 2.4$	$52.6 \pm 3.0$	$5.0 \pm 0.4$	$4.1 \pm 0.5$	$4.3 \pm 0.6$
7	Infected	$53.7 \pm 4.9$	$34.5 \pm 4.9$	$4.6 \pm 0.4$	$2.6 \pm 0.4$	$4.8 \pm 0.5$
7	Uninfected	$46.4 \pm 3.2$	$39.7 \pm 3.6$	$3.9 \pm 0.4$	$4.0 \pm 0.6$	$5.9 \pm 0.7$
11	Infected	$47.1 \pm 5.6$	$37.1 \pm 5.6$	$4.2 \pm 0.3$	$5.7 \pm 0.7^a$	$6.2 \pm 0.8$
11	Uninfected	$54.5 \pm 3.3$	$29.8 \pm 3.0$	$4.7 \pm 0.3$	$3.2 \pm 0.5^b$	$6.4 \pm 2.2$

<sup>1</sup>Each treatment comprised 3 poult from four cages.

<sup>2</sup>Concentrations of individual WBC populations were determined using concentration estimates of total WBC based on CELL-DYN automated hematology analysis and the percentages of

individual cell populations determined manually using Wright stained blood smears and a bright field microscope (1000X).

<sup>3</sup>Values in a column with no common superscript for the day of infection differ significantly ( $P \leq 0.05$ ).

**Table 5. Mean concentrations and ratio of CD4+ and CD8+ lymphocytes in peripheral blood of turkeys at different times following infection with  $12.5 \times 10^3$  oocysts of *E. adenoeides* at 20 d of age.**

Day of infection	Treatment <sup>1</sup>	Lymphocyte concentration (x $10^6/\text{mL}$ ) <sup>1,2</sup>		CD4+ to CD8+ lymphocyte ratio <sup>2,3</sup>
		CD4+	CD8+	CD4+ : CD8+
0	Infected	$9.0 \pm 1.4$	$2.5 \pm 0.6$	$4.2 \pm 0.5$
0	Uninfected	$7.0 \pm 0.5$	$2.1 \pm 0.6$	$3.4 \pm 0.3$
4	Infected	$4.1 \pm 0.5$	$0.6 \pm 0.1^b$	$7.2 \pm 0.9^a$
4	Uninfected	$5.6 \pm 0.5$	$1.4 \pm 0.1^a$	$4.0 \pm 0.3^b$
7	Infected	$3.7 \pm 0.7$	$2.2 \pm 0.2$	$1.7 \pm 0.3$
7	Uninfected	$2.2 \pm 0.3$	$1.8 \pm 0.2$	$1.4 \pm 0.2$
11	Infected	$4.1 \pm 0.6^a$	$1.4 \pm 0.2^a$	$3.6 \pm 0.6^a$
11	Uninfected	$1.9 \pm 0.2^b$	$0.9 \pm 0.1^b$	$2.1 \pm 0.1^b$

<sup>1</sup>Each treatment comprised 3 poult from four cages.

<sup>2</sup>The concentrations of CD4- and CD8-defined lymphocyte populations is based on the percentage of CD4+ and CD8+ lymphocytes in the small mononuclear cell population determined by immunofluorescent staining and population analysis by flow cytometry, and the concentration of lymphocytes in whole blood. The ratio between CD4+ and CD8+ lymphocytes was determined by dividing the percentage of CD4+ cells by the percentage of CD8+ cells.

<sup>3</sup>Values in a column with no common superscript for the day of infection differ significantly ( $P \leq 0.05$ ).

**Table 6. Weight gain and oocyst production of 20 d old turkey poult s infected with 12.5 x 10<sup>3</sup> oocysts of *E. adenoeides* and challenged at 34 d of age with 5 x 10<sup>4</sup> oocysts**

Treatment number <sup>1</sup>	Primary infection	Challenged	Weight gain (g) <sup>2</sup>	Oocysts/poult (x 10 <sup>-6</sup> ) <sup>3</sup>		
				5-6	6-7	7-8
1A	Yes	Yes	480 ± 27 <sup>a</sup>	12 ± 2	7 ± 2	3.2 ± 0
1B	Yes	No	535 ± 29 <sup>a</sup>	0	0	0
2A	No	Yes	307 ± 28 <sup>b</sup>	159 ± 13	55 ± 2	10 ± 3
2B	No	No	579 ± 7 <sup>a</sup>	0	0	0
P-value			0.005	Not done		

<sup>1</sup>Each treatment comprised two cages of 9 poult s.

<sup>2</sup>Mean weight gain ± SEM from d 0-6 after the challenge infection. Values in a column with no common superscript differ significantly.

<sup>3</sup>Oocysts produced/poult ± SEM from d 5-6, 6-7, and 7-8 after the challenge infection.





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## MEMORANDUM

TO: H. D. Chapman  
FROM: Craig N. Coon, Chairman  
Institutional Animal Care  
And Use Committee  
DATE: October 15, 2009  
SUBJECT: IACUC PROTOCOL APPROVAL  
Expiration date : October 31, 2012

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol #10011-  
“**DEVELOPMENT OF IMMUNITY TO EIMERIA ADENOEIDES IN THE TURKEY**”. You may  
begin this study immediately.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any  
changes in the protocol during the research, please notify the IACUC in writing **prior** to initiating the  
changes. If the study period is expected to extend beyond **10-31-2012**, you must submit a new protocol.  
By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for  
research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian



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Attn University of Arkansas Graduate School

June 20, 2013

Dear Sir,

I attest that Ujvala Deepthi Gadde was first author of the manuscript cited below and completed at least 51% of the work for the paper.

Gadde, U., H. D. Chapman, T. Rathinam, and G. F. Erf. 2009. Acquisition of Immunity to the Protozoan Parasite *Eimeria adenoeides* in Turkey Poults and the Peripheral Blood Leukocyte Response to a Primary Infection. Poult. Sci. 88:2346-2352.

Yours Sincerely,

H. David Chapman,  
University Professor,  
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### **CHAPTER III**

#### **CELLULAR IMMUNE RESPONSES, CHEMOKINE, AND CYTOKINE PROFILES IN TURKEY POULTS FOLLOWING INFECTION WITH THE INTESTINAL PARASITE *EIMERIA ADENOEIDES***

**Cellular Immune Responses, Chemokine, and Cytokine Profiles in Turkey Poult following Infection with the Intestinal Parasite *Eimeria adenoeides***

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## ABSTRACT

Cellular immune responses, chemokine, and cytokine profiles were investigated in 20 d old turkey poult following an oral infection with  $12.5 \times 10^3$  oocysts of *E. adenoides*, a protozoan parasite of the genus *Eimeria* that develops in the ceca. Large numbers of oocysts were produced in the feces of infected birds from d 5 after infection followed by a rapid decline by d 7. Local immune activities were characterized by observing the extent of leukocyte infiltration in the ceca by histology, measuring subsets of the lymphocyte population by immunohistochemistry, and determining the relative expression of cytokines by real-time RT-PCR. Inflammation, assessed by scoring the extent of cellular infiltration of leukocytes in sections of ceca, was significantly higher in infected poult compared to uninfected poult on d 4, 7, 9 and 11 following infection. The percent area occupied by CD4<sup>+</sup> and CD8<sup>+</sup> cells in the ceca was significantly greater on d 9 and 11 (CD4<sup>+</sup>) and d 11 (CD8<sup>+</sup>) in infected poult compared to uninfected controls. The relative expression of the chemokine CXCLi2 and the cytokines IL1 $\beta$ , IFN $\gamma$ , IL13 and IL10 was investigated in tissue samples taken from the ceca. Increased expression of CXCLi2 occurred on d 4 and d 7. Increased expression of IL10 and IFN $\gamma$  occurred on d 4, and IL1 $\beta$  and IL13 on d 7 post-infection. The increased leukocyte infiltration in the ceca, alterations in the lymphocyte subpopulations, and changes in expression of chemokines and cytokines are an indication of the cell mediated immune mechanisms occurring in the host as a result of exposure to *E. adenoides*.

**Key words:** *Eimeria*, turkey, immunity, CD4<sup>+</sup>, CD8<sup>+</sup> lymphocyte, cytokine

## INTRODUCTION

Coccidiosis is a widespread disease of the intestines of poultry caused by protozoan parasites of the genus *Eimeria*. In the turkey, acquisition of immunity plays an important role in the control of the disease and yet almost nothing is known regarding the mechanisms of immunity in this host (Chapman, 2008). Previously, it was shown that turkey poultts infected with  $12.5 \times 10^3$  oocysts of *E.adenoeides* develop immunity to a challenge infection given at 34 days of age (Gadde et al., 2009). Significant elevations in white blood cell concentrations in peripheral blood were demonstrated following infection. Changes in the concentration of CD4+ and CD8+ lymphocyte subpopulations in peripheral blood were also shown in infected poultts compared with uninfected control poultts. It was concluded that during the course of a primary infection of *E. adenoeides*, immune activities are initiated that are observable in the peripheral circulation and involve components of both innate and adaptive immunity.

In this study we further investigate local cellular immune activities in the ceca of young turkeys following a primary infection with *E. adenoeides*. We examine the extent of leukocyte infiltration in hematoxylin and eosin stained cecal sections, and characterize subsets of lymphocytes in the cecum by immunohistochemistry. Moreover, to gain insight into local innate and adaptive immune activities, we use real-time reverse transcription polymerase chain reaction to investigate changes in the relative gene expression of the chemokine CXCLi2, and the cytokines IL1 $\beta$ , IFN $\gamma$ , IL13 and IL10 in tissue samples taken from the ceca of turkeys following infection with *E. adenoeides*.

## MATERIALS AND METHODS

### *Birds and Husbandry*

Sixty female turkey poult (Nicholas 88) were used in the experiment. They were obtained from a local hatchery and reared in a Petersime brooder in an isolation building at the University of Arkansas at a stocking density of 257 cm<sup>2</sup> / poult and a brooder temperature of 35 to 38°C. At 17 d of age, all poult were identified with a unique wing band number and randomly allocated to six clean cages (10 poult / cage) in each of two separate battery units. Their husbandry followed procedures previously described (Gadde et al., 2009). This study was approved by the University IACUC committee and followed FASS guidelines for agricultural research.

### *Experimental Design*

The experimental design involved two treatments, poult infected with *E. adenoeides* and uninfected controls. Each treatment comprised three cages of ten poult. Cages containing poult of each treatment were kept in different battery units to reduce the possibility of accidental transfer of oocysts from infected to uninfected birds. Commencing on d 5 after infection and continuing to experiment termination on d 11, poult were transferred daily to an adjacent cage that had been thoroughly cleaned. This procedure further reduced the likelihood of reinfection with new oocysts produced during the acute phase of the life cycle.



## ***Infection***

The oocysts of *E. adenoeides* were propagated two weeks before the experiment and doses for infection prepared according to standard methods (Shirley, 1995). Three d after placement in cages, at 20 d of age, all poult were weighed individually and either inoculated orally with  $12.5 \times 10^3$  oocysts of *E. adenoeides* in 1 mL of water or given a sham dose of water (uninfected controls). This dose has been shown to cause depression of weight gain without mortality (Gadde et al., 2009). Feces from each cage were collected daily and the numbers of oocysts present determined (Shirley, 1995). Results were expressed as the total number of oocysts produced per poult for the 24 h period of collection.

## ***Tissue Collection***

Immediately prior to infection (d 0) and on d 4, 7, 9, and 11 after infection, two poult were randomly selected from each cage (6 poult / treatment), weighed, and killed by inhalation of carbon dioxide gas. The ceca were collected and rinsed thoroughly in Dulbecco's phosphate buffered saline (**DPBS**; Sigma Chemical Company, St. Louis, MO) to remove as much material from the lumen as possible. Two 1 cm sections were taken from the mid-part and blind-end of each cecum. They were fixed in 10% buffered formalin for histological examination or placed in cold DPBS until snap frozen in OCT (Tissue-Tek®, Sakura Finetek Inc., Torrance, CA) in liquid nitrogen for immunohistochemistry. An additional sample was taken from the middle of one cecum, collected in RNA preservation buffer (RNAlater®, Applied Biosystems, Foster City, CA) and stored at  $-20^{\circ}\text{C}$  until used for isolation of RNA and gene expression analysis.

### ***Leukocyte Infiltration***

Samples from the mid-part and blind-end of one of the ceca were embedded in paraffin and sections (one section from each part), about 5  $\mu\text{m}$  thick, stained with hematoxylin and eosin. Stained tissues were examined at 100 times magnification with a bright field microscope. An indication of the cellular infiltration in response to infection was obtained by subjectively assessing the extent and nature of leukocyte infiltration into the mucosa and submucosa of the entire section, requiring evaluation of 8-10 microscope fields per section. A numerical score was assigned where 0, 1, 2, and 3 represented no leukocyte infiltration, mild, moderate, or severe infiltration, respectively. The nature of the leukocyte infiltrate was characterized as mononuclear, granulocytic, or a mixture of granulocytes and mononuclear cells based on the distinct morphological characteristics of these cell types.

### ***Immunohistochemistry***

Cross-sections of 6  $\mu\text{m}$  thickness were obtained from frozen cecum samples, using a cryostat at -24°C (Leica CM3050, Leica Microsystems, Inc., Bannockburn, IL). The sections were mounted on poly-lysine coated slides, fixed in acetone for 5 minutes, and air dried. They were then incubated overnight with DPBS containing 10% horse serum to block nonspecific binding sites. After overnight incubation, the sections were washed with DPBS and incubated for 30 minutes with primary antibodies. The antibodies used were unlabeled mouse anti-chicken CD4 (clone CT-4; IgG1) and mouse anti-chicken CD8 $\alpha$  (clone 3-298; IgG1) antibodies (Southern

Biotechnology Associates Inc., Birmingham, AL) known to cross-react with turkey CD4 and CD8, respectively (Li et al., 1999). After 30 minutes of incubation, the sections were washed to remove any unbound primary antibody and then incubated for 30 minutes with a secondary antibody (biotinylated horse anti-mouse IgG) (Vector Laboratories Inc., Burlingame, CA). The sections were washed once more and then incubated for 30 minutes with avidin-biotin peroxidase complex (Vectastain Elite ABC reagent, Vector Laboratories Inc., Burlingame, CA). Binding of the ABC reagent to the biotinylated secondary antibody was detected by incubating the sections with 3, 3'-diaminobenzidine (DAB, Sigma Chemical Co., St. Louis, MO). The sections were washed and then counterstained with Harris Hematoxylin (Electron Microscopy Sciences, Hatfield, PA) for 30 seconds, dehydrated and covered with water soluble mounting medium (Aquamont, Thermo Shandon Inc., Lerner Laboratories, Pittsburgh, PA). Staining controls for both primary and secondary antibody were included by incubating the sections with an isotype control (IgG1 isotype control from murine myeloma; Sigma Chemical Company, St. Louis, MO) instead of specific primary antibody or without primary antibody, respectively. The stained sections were analyzed using Image-Pro Plus 6.2 (Media Cybernetics, Silver Springs, MD) connected to an Olympus B x 50 light microscope (Olympus, Center Valley, PA) at 100 x magnification. The percentage area (% area) occupied by immunostained (brown) cells (CD4+ lymphocytes and CD8+ lymphocytes) in each of the sections from the two parts of the cecum was estimated.

#### ***Isolation of RNA, Reverse Transcription, and Real-time PCR***

Total RNA was isolated from the cecum samples stored in RNAlater®, using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Approximately 30 mg of cecal tissue was homogenized in lysis

buffer and RNA isolated following manufacturer's instructions. An additional DNase digestion step was included to remove any contaminating DNA. The quantity of RNA was assessed by spectrophotometry (Genesys 10, Thermospectronic, Rochester, NY) measuring absorbance at 260 nm and checked for purity by measuring its OD<sub>260</sub> / OD<sub>280</sub> ratio. RNA (eluted in 30 µL RNase free water) was stored at -80°C until required for reverse transcription to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). For reverse transcription of RNA, the reaction mixture comprised about 2 µg of RNA per sample, 4 µL of 10 x RT buffer, 1.6 µL of 25 dNTP mix (100 mM), 4 µL of 10 x RT random primers, 2 µL of Multiscribe Reverse Transcriptase (50 U/µL), and nuclease free water to make up a final volume of 40 µL. The reaction was carried out in a thermal cycler (Genius Thermal Cycler, Techne Inc., Princeton, NJ); cycling conditions were 25 °C for 10 min, 37 °C for 2 h, and 85 °C for 5 min. The cDNA samples were aliquoted and stored at -20 °C.

Differential expression of the chemokine CXCLi2, and cytokines IL1β, IFNγ, IL13 and IL10 in the cDNA samples was assessed by real-time PCR using an Applied Biosystems 7300 sequence detection system (Applied Biosystems, Foster City, CA). Previously published primer and probe sequences specific for the chicken and turkey, or turkey only, were used (Mayne et al., 2007). These sequences are shown in Table 1. All probes were labeled with 6-carboxyfluorescein at the 5' terminus and 6-carboxytetramethylrhodamine at the 3' terminus. The reaction mixture for each sample comprised 2 µL of cDNA, 12.5 µL of Taqman universal PCR master mix (Applied Biosystems), 200 nM forward primer, 200 nM reverse primer, 100 nM probe, and nuclease free water to make up a final volume of 25 µL. The cycling conditions used were one cycle at 50 °C for 2 min, one cycle at 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All the samples were analyzed in triplicate for target genes and in duplicate

for the endogenous control (28sRNA). Negative controls were included to check for non-specific amplification of primers and probes. A calibrator sample (a pool of cDNA from uninfected birds on d 0) was also included in each plate. Differences in the relative expression of the target genes were quantified by the delta delta  $C_T$  ( $2^{-\Delta\Delta C_T}$ ) method described by Livak and Schmittgen (2001).

### ***Data Analysis***

Statistical analysis was carried out using JMP software (version 8.0.2; SAS Institute Inc., Cary, NC). All the data were expressed as mean  $\pm$  SEM for each treatment. Due to the presence of interactions between the two parameters (day and treatment) in two-way ANOVA, the data were analyzed by one-way ANOVA to compare the two treatments on each day. For day-wise comparison within each treatment, the data were analyzed by one-way ANOVA and the means separated by Tukey's HSD test. Results were considered to be significantly different if  $P \leq 0.05$ .

## **RESULTS**

### ***Oocyst Production***

The time course of appearance of oocysts in the feces following infection is shown in Fig.1. Oocysts were first produced from d 4-5; numbers peaked by d 5-6 and subsequently rapidly declined by d 7. Small numbers of oocysts were still being produced from d 10-11.

### ***Leukocyte Infiltration***

No difference was observed in sections taken from the mid-part and blind-end of the cecum and therefore their scores were combined. Little infiltration, if any, was observed in uninfected poult or infected poult on d 0 (Table 2). Cellular infiltration, consisting especially of mononuclear cells, was observed in infected poult and was significantly higher when compared to controls on d 4, 7, 9 and 11 after infection. Compared to d 0, infiltration in the ceca of infected poult was significantly higher at all time points examined (Table 2). Infiltration was highest on d 4 followed by a subsequent decline.

### ***Immunohistochemistry***

The data for the mid-part and blind-end of the ceca were combined as there were no differences observed between the two parts. In both the uninfected and infected poult, the percent area occupied by CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes in the mucosa and submucosa increased with age of the poult. However, compared to uninfected poult, the percent area occupied by CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes in infected poult was higher ( $P < 0.05$ ) on day 9 and 11 (CD4) and on day 11 (CD8) of infection, respectively (Table 3). While the percent area estimates of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes were based on examination of the entire mucosa and submucosa of the tissue sections, stained lymphocytes were primarily located in the lamina propria and epithelium.

### ***Real-Time PCR***

Mean fold changes in chemokine and cytokine expression in comparison to the calibrator are presented in Table 4. The mean fold change of CXCLi2 in infected poult was significantly increased on d 4 and 7 compared to uninfected poult. The expression of IL1 $\beta$  and IL13 was significantly elevated in infected poult on d 7, expression of IL10, and IFN $\gamma$  was elevated on d 4 compared to uninfected poult (Table 4).

No differences were observed in the relative gene expression of all the genes observed on different days of infection in uninfected poult. The relative expression of CXCLi2, IFN $\gamma$  and IL10 in the infected poult was significantly higher on d 4 of infection, and that of IL13 higher on d 7 of infection compared to all the other days of infection. No differences were seen in the relative expression of IL1 $\beta$  in infected poult on different days of infection.

## **DISCUSSION**

*Eimeria adenoeides* is highly pathogenic causing very high mortality and reduced weight gain in young turkey poult (Clarkson, 1958). An objective of this study was to investigate inflammatory responses and cellular immune activities occurring in the cecum, the site of multiplication of the parasite. Rigorous procedures were adopted to prevent unintentional re-infection and therefore the oocyst output and infection associated immune activities observed were a consequence of the inoculum of oocysts given to the poult. In other published studies no

obvious precautions to prevent reinfection, a prerequisite for investigating responses to a primary infection, were carried out.

An indication of the inflammatory response to infection was obtained by assessing leukocyte infiltration into the mucosa and submucosa of the ceca. Infiltration with mononuclear leukocytes (lymphocytes and macrophages) was significantly elevated in infected poult and remained high throughout the course of infection indicating the initiation of immune activities in the host. Infiltration of inflammatory cells into the intestinal mucosa and production of inflammatory mediators is an important component of intestinal immunity and occurs during infection and repair of damaged tissue (Nathan, 2002; Bourlioux et al., 2003).

Characterization of the cellular immune response at local sites of parasite development is important in order to understand mechanisms of cell mediated immunity (Lillehoj and Lillehoj, 2000). In this study, T cell distribution was investigated by immunohistochemical staining of cecal tissue. The percent area occupied by CD4+ cells (T helper cells) was significantly higher on d 9 and 11 in infected poult than in uninfected poult. The percent area occupied by CD8+ cells was elevated on d 11 in infected poult. An increase in the area occupied by T helper and cytotoxic lymphocytes suggests greater numbers of these cells at the site of parasite multiplication and indicates initiation of specific effector cell mediated immune activities against parasitic stages in intestinal tissue. While specific subsets of T cells were found elevated in stained sections only from d 9, increased infiltration of mononuclear leukocytes was observed from d 4 following infection. The leukocytes infiltrating cecal tissue on d 4 and d 7 may be mostly macrophages, along with CD4- and CD8-negative lymphocytes such as  $\gamma\delta$  T cells and B cells that were recruited to the mucosa due to the inflammatory activities initiated by infection. On the other hand, the infiltration of CD4+ and CD8+ lymphocytes observed on d 9 and 11



post-infection may mark the arrival of effector lymphocytes (T helper and cytotoxic, respectively). Similarly, a biphasic increase in numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes was observed in the epithelium and lamina propria of the small intestine of chickens following infection with *E. maxima* (Rothwell et al., 1995).

An objective of this study was to investigate gene expression of various chemokines and inflammatory cytokines in infected ceca. As antibodies to turkey cytokines and chemokines were not available, the relative gene-expression was investigated at the transcriptome-, rather than, the protein-level. Chemokines, such as CXCLi2, act as mediators for leukocyte recruitment during the course of inflammation in *Eimeria* infections (Hong et al., 2006b). The relative expression of CXCLi2 was found to be significantly increased in infected poult on d 4 and d 7, which is consistent with the recruitment of mononuclear leukocytes in the ceca during the acute phase of the infection. In the chicken, expression of chemokine IL8 was found to be increased following infection with *E. tenella*, *E. maxima*, and *E. acervulina* (Laurent et al., 2001; Swinkels et al., 2006; Cornelissen et al., 2009), attesting to similar leukocyte recruitment efforts during *Eimeria* infection.

IL1 $\beta$  is an important inflammatory cytokine, most commonly produced by activated macrophages, that plays an important role in the innate immune response and helps in recruitment of inflammatory cells to the site of infection (Hong et al., 2006b). In this study, expression levels of IL1 $\beta$  were elevated but due to high variability, significant differences between infected and uninfected controls were only observed on d 7. In chickens infected with *E. tenella* or *E. maxima* an approximately 80- and 27-fold change in IL1 $\beta$  expression occurred in the cecum and jejunum, respectively, following infection. In another study in chickens,

intestinal intraepithelial lymphocyte gene transcripts for IL1 $\beta$  were up-regulated following infection with *E. acervulina* or *E. tenella* (Hong et al., 2006a).

IFN $\gamma$  is a signature cytokine for cell-mediated immune responses. It is produced primarily by CD4<sup>+</sup> type-1 T helper (**Th1**) cells, activated cytotoxic T cells and natural killer (**NK**) cells. IFN $\gamma$  plays an important role in the activation of macrophages harboring intracellular pathogens, enhancement of antigen presentation and differentiation of Th1 cells (Wigley and Kaiser, 2003). The expression of IFN $\gamma$  was significantly elevated on d 4 suggesting macrophage activation and amplification of the inflammatory response. Several studies have shown an increase in IFN $\gamma$  expression in chickens infected with *Eimeria* (Yun et al., 2000; Laurent et al., 2001; Hong et al., 2006a, b; Cornelissen et al., 2009). In the current study, the early rise in IFN $\gamma$  expression is likely due to local intra-epithelial lymphocytes and possibly NK cells (Abbas et al., 2010). With the arrival of CD4<sup>+</sup> and CD8<sup>+</sup> effector lymphocytes, the relative IFN $\gamma$  expression levels appear to rise again on d 11, although due to high variability this increase was not significant.

IL13 is produced mainly by CD4<sup>+</sup> type 2 T helper (**Th2**) cells in addition to epithelial cells (Hong et al., 2006b) and has a major role in isotype switching by B cells. In this study, a significant increase in expression of IL13 was observed on d 7 following infection. In infected poult, the expression level was significantly higher on d 7 compared to the levels on all the other days following infection. The peak IL13 expression by d 7, like that of IFN $\gamma$ , occurs when peak inflammatory activity was observed in the mucosa. IL13 production at this time is likely due to intra-epithelial lymphocytes as well as mucosal epithelial cells and mast cells (Abbas et al., 2010). The observation that the increase in CD4<sup>+</sup> lymphocytes on d 9 and 11 was not

accompanied by an increase in IL13 expression supports development of a Th1- rather than a Th2-adaptive response to *Eimeria* infection.

IL10 is an important regulatory cytokine produced by T cells and macrophages, its major biological function is the control of innate immune responses and cell-mediated immunity. IL10 is considered to play a role in preventing the development of Th1 cytokines in chickens (Rothwell et al., 2004), but an immunostimulatory role has been described recently wherein IL10 is known to induce IFN $\gamma$  production by NK and cytotoxic T cells (Shibata et al., 1998; Abdul-Careem et al., 2008). In this study, the level of IL10 expression was found to be significantly elevated on d 4 following infection when mononuclear cell infiltration was already substantially higher in infected poult than controls. While the role of this cytokine early in infection is not understood at this time, there are a few reports of IL10 expression in chickens infected with *Eimeria* species (Hong et al., 2006b; Cornelissen et al., 2009).

Overall, the infiltration of mononuclear leukocytes, including T helper and cytotoxic lymphocytes, together with the observed cytokine expression patterns in the ceca indicate that a primary *E. adenoeides* infection in the turkey stimulates inflammatory and cell mediated immune activities at the site of infection. The cytokine expression profiles during the early phase of *Eimeria* infection may be attributed to inflammatory activities of local macrophages, injured tissue cells, mast cells, NK cells, IEL and epithelial cells as well as recruited macrophages and lymphocytes. The arrival of CD4+ and CD8+ lymphocytes on d 9 and 11, together with a variable increase in IFN $\gamma$  expression, appears to mark the adaptive phase of the response to *Eimeria* infection in the cecum.

In this study, we document inflammatory and cellular immune activities and cytokine profiles in ceca of turkeys following a primary infection with *E. adenoeides*, one of the most

pathogenic species of *Eimeria* that parasitizes the intestine of this host. Alteration in lymphocyte subpopulations and expression of a chemokine and cytokines has not previously been reported for turkeys infected with *Eimeria*.

## ACKNOWLEDGEMENTS

We would like to thank Fengying Shi for her advice concerning the techniques of immunohistochemistry and the two-step real time RT-PCR procedures.

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**Table 1.** Primer and probe sequences used for Real Time PCR

Target <sup>1</sup>	Primer / probe sequence <sup>2</sup>	Accession no.
28S	Chicken and turkey	X59733
Forward primer	5'-GGCGAAGCCAGAGGAAACT-3'	
Reverse primer	5'-GACGACCGATTTCACGTC-3'	
Probe	5'-AGGACCGCTACGGACCTCCACCA-3'	
CXCLi2	Turkey	AM493430
Forward primer	5'-TGGCTCTCCTCCTGGTTTCA-3'	
Reverse primer	5'-GCAGCTCGTTCCCCATCTT-3'	
Probe	5'-CAGCTCTGTCAACAAGGTAGGACCCTGGT-3'	
IL1 $\beta$	Chicken and turkey	AJ245728
Forward primer	5'-GCTCTACATGTCGTGTGTGATGAG-3'	
Reverse primer	5'-TGTCGATGTCCCGCATGA-3'	
Probe	5'-CCACACTGCAGCTGGAGGAAGCC-3'	
IFN $\gamma$	Turkey	AJ000725
Forward primer	5'-AACCTTCCTGATGGCGTGAA-3'	
Reverse primer	5'-CTTGCGCTGGATTCTCAAGTC-3'	
Probe	5'-AAAGATATCATGGACCTGGCCAAGCTTCA-3'	
IL13	Turkey	AM493431
Forward primer	5'-CCTGCACGGCCAGATGA-3'	
Reverse primer	5'-GGCAAGAAGTTCCGCAGGTA-3'	
Probe	5'-TGCCAGCTGAGCACCGACAACG-3'	
IL10	Turkey	AM493432
Forward primer	5'-CGACCTGGGCAACATGCT-3'	
Reverse primer	5'-CCTCTCGCAGGTGAAGAAGTG-3'	
Probe	5'-CCTGAAGATGACAATGAAGCGCTGTCA-3'	

<sup>1</sup>IL = Interleukin; IFN = Interferon

<sup>2</sup>Sequences from Mayne et al. (2007)

**Table 2.** Score for severity of leukocyte infiltration in sections from the mid-part and blind-end of ceca from turkey poult infected with *E. adenoeides*

Treatment <sup>1</sup>	Mean score-severity of leukocyte infiltration in cecum <sup>2</sup>				
	Day 0	Day 4	Day 7	Day 9	Day 11
Uninfected	0.0 ± 0.0	0.2 ± 0.2 <sup>b</sup>	0.0 ± 0.0 <sup>b</sup>	0.2 ± 0.2 <sup>b</sup>	0.3 ± 0.3 <sup>b</sup>
Infected	0.0 ± 0.0 <sup>z</sup>	2.7 ± 0.2 <sup>a,x</sup>	2.0 ± 0.2 <sup>a,xy</sup>	1.8 ± 0.3 <sup>a,y</sup>	1.7 ± 0.2 <sup>a,y</sup>

<sup>a,b</sup> For each day, values in a column with no common superscript differ significantly ( $P \leq 0.05$ )

<sup>x,y,z</sup> For each treatment, values in a row with no common superscript differ significantly ( $P \leq 0.05$ )

<sup>1</sup>The poult at 20 d of age were infected with  $12.5 \times 10^3$  oocysts of *E.adenoeides* or given sham dose of water. They were moved to clean cages daily from d 5 of infection, to prevent re-infection with oocysts that were shed in the feces.

<sup>2</sup>Leukocyte infiltration was scored on a 0-3 scale where 0, 1, 2, and 3 represented no infiltration, mild, moderate, or severe infiltration, respectively. One section from each part was used for scoring. An average of 8-10 fields were observed for each section and infiltration characterized as mononuclear, granulocytic or a mixture of both. A numerical score was assigned to each section. Results for the mid-part and blind-end of each cecum were combined. For each treatment, values are the mean ± SEM for six poult.



**Table 3.** Percent area occupied by CD4+ and CD8+ lymphocytes in sections taken from the mid-part, or blind-end of ceca from turkey poult s infected with *E. adenoeides*

Day	Percent area occupied in cecum (Mean % $\pm$ SEM) <sup>2</sup>			
	CD4+ lymphocytes		CD8+ lymphocytes	
	Uninfected <sup>1</sup>	Infected <sup>1</sup>	Uninfected	Infected
0	0.57 $\pm$ 0.09 <sup>y</sup>	0.58 $\pm$ 0.06 <sup>z</sup>	1.27 $\pm$ 0.15 <sup>y</sup>	1.33 $\pm$ 0.13 <sup>z</sup>
4	0.73 $\pm$ 0.13 <sup>xy</sup>	0.79 $\pm$ 0.08 <sup>yz</sup>	1.62 $\pm$ 0.14 <sup>xy</sup>	1.61 $\pm$ 0.06 <sup>yz</sup>
7	0.88 $\pm$ 0.13 <sup>xy</sup>	0.97 $\pm$ 0.05 <sup>xy</sup>	1.75 $\pm$ 0.08 <sup>xy</sup>	1.87 $\pm$ 0.05 <sup>xy</sup>
9	0.93 $\pm$ 0.05 <sup>xy,b</sup>	1.21 $\pm$ 0.05 <sup>x,a</sup>	2.00 $\pm$ 0.09 <sup>x</sup>	2.05 $\pm$ 0.11 <sup>x</sup>
11	1.05 $\pm$ 0.11 <sup>x,b</sup>	1.59 $\pm$ 0.06 <sup>w,a</sup>	1.55 $\pm$ 0.12 <sup>x,b</sup>	1.98 $\pm$ 0.07 <sup>xy,a</sup>

<sup>a,b</sup> For each day, values in a row (under each subset of lymphocytes) with no common superscript show significant differences ( $P \leq 0.05$ ).

<sup>w,x,y,z</sup> For each treatment, values in a column with no common superscript differ significantly ( $P \leq 0.05$ ).

<sup>1</sup>The poult s at 20 d of age were infected with  $12.5 \times 10^3$  oocysts of *E.adenoeides* or given sham dose of water. They were moved to clean cages daily from d 5 of infection, to prevent re-infection with oocysts that were shed in the feces.

<sup>2</sup>The percent area occupied by CD4+ and CD8+ lymphocytes was obtained by indirect immunohistochemical staining of cecal sections using anti-chicken CD4 (clone CT-4) and anti-chicken CD8 $\alpha$  (clone 3-298) specific mouse monoclonal antibodies (Southern Biotechnology

Associates Inc., Birmingham, AL) and Vectastain Elite secondary reagents (Vector Laboratories Inc., Burlingame, CA). For each treatment, values are the mean  $\pm$  SEM for six poult.

**Table 4.** Fold change in relative gene expression of a chemokine and cytokines in the ceca of poult s infected with *E. adenoeides* compared with uninfected controls

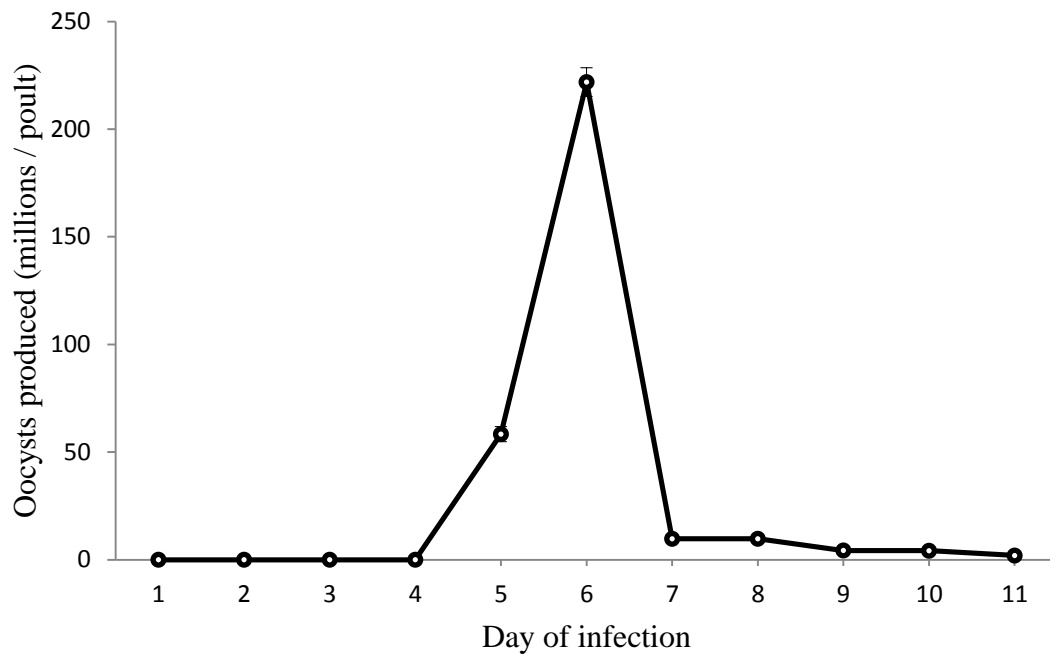
Gene	Treatment <sup>1</sup>	Fold change (Mean $\pm$ SEM) <sup>2</sup>				
		Day 0	Day 4	Day 7	Day 9	Day 11
CXCLi2	Uninfected	0.46 $\pm$ 0.16	2.90 $\pm$ 1.10 <sup>b</sup>	0.38 $\pm$ 0.20 <sup>b</sup>	2.56 $\pm$ 1.12	1.30 $\pm$ 0.50
	Infected	0.56 $\pm$ 0.19 <sup>y</sup>	21.54 $\pm$ 3.14 <sup>a,x</sup>	1.28 $\pm$ 0.31 <sup>a,y</sup>	5.16 $\pm$ 2.85 <sup>y</sup>	3.64 $\pm$ 2.46 <sup>y</sup>
IL1 $\beta$	Uninfected	2.27 $\pm$ 0.91	1.98 $\pm$ 0.37	1.96 $\pm$ 0.74 <sup>b</sup>	3.59 $\pm$ 0.72	3.89 $\pm$ 2.50
	Infected	0.69 $\pm$ 0.11	12.14 $\pm$ 6.52	6.20 $\pm$ 1.35 <sup>a</sup>	17.43 $\pm$ 7.17	10.98 $\pm$ 7.05
IFN $\gamma$	Uninfected	1.71 $\pm$ 0.31	2.34 $\pm$ 0.75 <sup>b</sup>	1.10 $\pm$ 0.67	1.45 $\pm$ 0.52	0.94 $\pm$ 0.68
	Infected	1.85 $\pm$ 0.75 <sup>y</sup>	13.96 $\pm$ 2.53 <sup>a,x</sup>	3.08 $\pm$ 1.04 <sup>y</sup>	2.73 $\pm$ 0.94 <sup>y</sup>	4.55 $\pm$ 2.57 <sup>y</sup>
IL13	Uninfected	1.68 $\pm$ 0.46	3.31 $\pm$ 0.69	4.05 $\pm$ 1.14 <sup>b</sup>	4.73 $\pm$ 2.60	5.68 $\pm$ 1.33
	Infected	2.41 $\pm$ 0.92 <sup>y</sup>	4.34 $\pm$ 1.01 <sup>y</sup>	16.44 $\pm$ 3.24 <sup>a,x</sup>	7.98 $\pm$ 1.65 <sup>y</sup>	6.1 $\pm$ 2.18 <sup>y</sup>
IL10	Uninfected	0.73 $\pm$ 0.37	4.99 $\pm$ 1.28 <sup>b</sup>	1.59 $\pm$ 0.62	3.89 $\pm$ 1.52	2.46 $\pm$ 1.26
	Infected	0.44 $\pm$ 0.16 <sup>y</sup>	15.28 $\pm$ 4.59 <sup>a,x</sup>	1.93 $\pm$ 0.79 <sup>y</sup>	4.91 $\pm$ 2.64 <sup>y</sup>	2.60 $\pm$ 1.33 <sup>y</sup>

<sup>a,b</sup>For each gene, values in a column with no common superscript differ significantly ( $P \leq 0.05$ )

<sup>x,y</sup> For each gene, values in a row with no common superscript differ significantly ( $P \leq 0.05$ ).

<sup>1</sup>The poult s at 20 d of age were infected with  $12.5 \times 10^3$  oocysts of *E.adenoeides* or given sham dose of water. They were moved to clean cages daily from d 5 of infection, to prevent re-infection with oocysts that were shed in the feces.

<sup>2</sup>Relative gene expression was measured by Real Time RT-PCR. The data were analyzed using the  $2^{-\Delta\Delta C_T}$  method and expressed as fold change in comparison to the calibrator sample. For each treatment, values are the mean  $\pm$  SEM for six poult.



Gadde et al., Legend to Figure 1.

Fig 1. Millions of oocysts produced in the feces / poult (Mean  $\pm$  SEM) from d 0-11 following infection with  $12.5 \times 10^3$  oocysts of *E. adenoeides*. The number of poults from which feces was collected varied on different days (N = 28 on d 1-4, 18 on d 5-7, 12 on d 8-9, 6 on d 10 & 11) as some of the poults were sacrificed for collection of tissue samples. Poults were moved to clean cages on a daily basis to prevent re-infection with oocysts passed out in the feces.



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## MEMORANDUM

TO: H. D. Chapman  
FROM: Craig N. Coon, Chairman  
Institutional Animal Care  
And Use Committee  
DATE: October 15, 2009  
SUBJECT: IACUC PROTOCOL APPROVAL  
Expiration date : October 31, 2012

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol #10011-  
“**DEVELOPMENT OF IMMUNITY TO EIMERIA ADENOEIDES IN THE TURKEY**”. You may  
begin this study immediately.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any  
changes in the protocol during the research, please notify the IACUC in writing **prior** to initiating the  
changes. If the study period is expected to extend beyond **10-31-2012**, you must submit a new protocol.  
By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for  
research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian



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Attn University of Arkansas Graduate School

June 20, 2013

Dear Sir,

I attest that Ujvala Deepthi Gadde was first author of the manuscript cited below and completed at least 51% of the work for the paper.

Gadde, U., H. D. Chapman, T. Rathinam, and G. F. Erf. 2011. Cellular Immune Responses, Chemokine, and Cytokine Profiles in Turkey Poult following Infection with the Intestinal Parasite *Eimeria adenoeides*. Poult. Sci. 90:2243-2250.

Yours Sincerely,

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## **CHAPTER IV**

### **ACQUISITION OF IMMUNITY TO THE PROTOZOAN PARASITE *EIMERIA ADENOEIDES* IN TURKEY POULTS AND CELLULAR RESPONSES TO INFECTION**

**Acquisition of Immunity to the Protozoan Parasite *Eimeria adenoeides* in Turkey Poult  
and Cellular Responses to Infection**

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## ABSTRACT

Newly hatched turkey poult s were infected with  $10^2$  oocysts of *Eimeria adenoeides* and subsequently re-infected with  $10^3$  and  $10^4$  oocysts at 6 and 12 d of age respectively. Three peaks in oocyst production were observed in the feces of poult s following this series of infections. A second group of poult s given the same dosing regimen was challenged with  $5 \times 10^4$  oocysts / poult at different times in order to evaluate the acquisition of immunity. Judged by weight gain and mortality, no protection had been acquired at 6 d of age, but partial protection was observed by 12 and 18 d of age. A third group of poult s were also infected with  $10^2$  oocysts and subsequently re-infected with  $10^3$  and  $10^4$  oocysts at 6 and 12 d of age in order to evaluate cellular immune responses to infection. Sections of ceca from infected poult s showed a significantly higher leukocyte infiltration on d 6, 10, 12, 16, and 18 after infection than uninfected controls. The percent area occupied by CD4+ and CD8+ lymphocytes in the ceca, as assessed by immunohistochemistry, was significantly elevated in infected poult s on d 12, 16 and 18. The relative expression of chemokine CXCLi2, and cytokines IL1 $\beta$ , IFN $\gamma$ , IL10, IL13, IL2, IL12b, and IL18 was measured by real-time reverse-transcription PCR. The expression of CXCLi2 and IL10 was found to be elevated on d 12, and IFN $\gamma$  on d 10, 12, and 16. Expression of IL13 and IL18 was increased on d 10 and IL2 on d 10 and 16, and that of IL12b on d16 in infected poult s. Increase in the infiltration of leukocytes, percent area occupied by CD4+ and CD8+ lymphocytes, and changes in the relative expression of cytokines in the ceca characterize the dynamics of immune responses in turkey poult s infected with *E. adenoeides* early in life.

**Key words:** *Eimeria*; turkey; oocyst; cellular immunity; lymphocyte; cytokine

## INTRODUCTION

Coccidiosis is an important disease of turkeys caused by protozoan parasites of the genus *Eimeria* (Chapman, 2008). One of the most prevalent species is *E. adenoeides* which develops in the ceca and is capable of causing reduced feed intake, decreased weight gain, poor feed conversion, and mortality if birds are exposed to large numbers of the infective transmission stage of the lifecycle, the sporulated oocyst (Clarkson, 1958). *E. adenoeides* is regarded as the most pathogenic species of *Eimeria* that parasitizes the turkey and oocysts of this species have been identified in most litter samples from turkey farms submitted to our laboratory (Rathinam and Chapman, 2009). Infection with this species is known to stimulate a protective immune response (Clarkson, 1958) but very little is known regarding the acquisition of immunity and the cellular mechanisms involved. Previously we infected turkey poults at 20 d of age with a high dose of oocysts ( $12.5 \times 10^3$  / poult) designed to cause significant pathology, and quantified cellular immune responses (CD4+ and CD8+ lymphocyte populations and chemokine, and cytokine expression profiles) in the ceca (Gadde et al., 2011). The changes noted were considered indicative of a significant inflammatory response to infection with this parasite.

In a commercial setting, vaccination of poultry against coccidiosis frequently involves administering low doses of oocysts in the hatchery (Chapman, 2000). The development of protective immunity requires this preliminary exposure to the parasite followed by secondary exposure to vaccinal oocysts once birds are placed upon litter. In this experiment we attempted to mimic commercial practice by infecting newly hatched poults with a low dose of oocysts ( $10^2$  oocysts of *E. adenoeides* / poult) upon arrival from the hatchery. We subsequently re-infected them with  $10^3$  and  $10^4$  oocysts when they were 6 and 12 d of age as could occur following

placement upon litter. The time course of oocyst excretion following this series of three doses was measured by counting the numbers of new oocysts shed in the feces. Poults were challenged with a high dose of oocysts at 6, 12, and 18 d of age to evaluate the extent of protection acquired as a result of exposure to infection. Tissue samples were taken from selected poults and lymphocyte populations and cytokine profiles determined in order to assess cellular immune responses to infection.

## **MATERIALS AND METHODS**

### ***Animals and Husbandry***

Female turkey poults (Nicholas 88) were obtained from a local hatchery approximately 8 hours after hatch and transferred to an isolation building at the University of Arkansas. They were allocated to preheated cages maintained at a brooding temperature of 35 to 38°C at an initial stocking density of 257 cm<sup>2</sup> / poult. An unmedicated turkey starter ration was provided and birds were given free access to water. Husbandry followed procedures previously described (Gadde et al., 2009). The study was approved by the University Institutional Animal Care and Use Committee and followed FASS guidelines for agricultural research (FASS, 1999).

### ***Parasite***

*E. adenoeides* has been maintained in the laboratory by periodical propagation of oocysts in 2 wk old turkey poults. The methods involved in the propagation and harvesting of the parasites, and

the preparation of oocysts for infection, have been described by Shirley (1995). Oocysts were less than 6 wk old when used in the experiments.

### ***Experimental design***

Three separate experiments were carried out using a total of 408 newly hatched turkey poults. In all three experiments poults were orally inoculated upon arrival from the hatchery (d 0) with  $10^2$  oocysts of *E. adenoeides* / poult and reinfected on d 6 and d 12 with  $10^3$  and  $10^4$  oocysts respectively. Uninfected poults were sham inoculated with water. The first experiment was designed to investigate parasite multiplication by determining the number of new oocysts produced in the feces of birds following this dosing regimen (oocyst excretion). The second experiment evaluated the extent of protection acquired following infection and the third experiment measured cellular immune responses.

### ***Oocyst excretion***

Upon arrival from the hatchery (d 0), 24 poults were allocated to three cages (8 poults / cage) and infected with three doses of oocysts as described above. A further 24 poults were allocated to three cages and served as uninfected controls. Feces were collected from trays every 3 d and the number of oocysts present counted as described by Shirley (1995).

### ***Acquisition of immunity***

A second group of 72 newly hatched poult were allocated to three cages (24 poult / cage) and given the dosing regimen described. These poult served as the infected challenged group (IC) for determination of protection. A further 144 poult were allocated to six cages (24 poult / cage) maintained in a separate isolation room to avoid accidental infection. They were not infected but given sham doses of water when 0, 6, and 12 d of age. These poult were to serve as an uninfected challenged group (UC) and an uninfected unchallenged group (UU) for immunity evaluation.

At 6, 12 and 18 d of age, eight poult were randomly selected from the three cages of the IC, UC, and UU groups respectively (24 poult / treatment), weighed and wing banded. They were then either challenged with  $5 \times 10^4$  oocysts (IC and UC groups) or given a sham dose of water (UU group). Six d later they were weighed again and weight gain from d 0-6 calculated. Mortality resulting from infection was determined.

### ***Cellular immune responses***

A third group of 72 poult were allocated to three cages (24 poult / cage). They were also infected on d 0 with  $10^2$  oocysts and re-infected on d 0 and d 6 with  $10^3$  and  $10^4$  oocysts / poult respectively. A further 72 poult were allocated to another three cages but were not infected; these birds were given a sham dose of water and served as uninfected controls. Cages of the uninfected controls were maintained in a separate isolation room in order to avoid the possibility of accidental infection.

Prior to infection on d 0, two birds were removed from each cage (six birds from the infected group and six from the uninfected controls), weighed, and killed by inhalation of carbon dioxide gas. These birds were used for tissue collection. This procedure was repeated on d 4, 6, 10, 12, 16, and 18 after infection on d 0.

***Tissue collection.*** Three 1-cm transverse sections were taken from the mid-part of the cecum of each poult as no differences in leukocyte infiltration were seen between mid-part and blind-end in our earlier experiments. They were thoroughly flushed with Dulbecco's phosphate buffered saline (DPBS; Sigma Chemical Company, St Louis, MO) to remove as much material from the lumen as possible. The first section was placed in 10% formalin for subsequent histological evaluation of leukocyte infiltration. The second section was placed in cold DPBS, snap frozen using OCT in liquid nitrogen, and stored at -80°C for immunohistochemistry. The third section was placed in RNA preservation buffer (RNAlater<sup>®</sup>, Applied Biosystems, Foster City, CA) and stored at -20°C for subsequent gene expression analysis.

***Leukocyte infiltration and immunohistochemistry.*** Procedures for determining leukocyte infiltration in the ceca have previously been described (Gadde et al., 2011). Briefly, formalin preserved sections were embedded in paraffin and 5µm sections stained with hematoxylin and eosin stain. They were examined at 100x magnification with a bright field microscope to ascertain the nature and extent of leukocyte infiltration in the mucosa and submucosa of entire section (6-10 microscopic fields). The nature of the infiltrate was characterized as mononuclear, granulocytic, or a mixture of mononuclear cells and granulocytes based on the morphological features of the infiltrating cells. An indication of extent of cellular infiltration was obtained by assigning a numerical score where 0, 1, 2, and 3 represented no, mild, moderate, or severe infiltration of the tissues respectively.



Full details of immunohistochemistry procedures and the suppliers of reagents used have been provided (Gadde et al., 2011). Cross sections (6  $\mu\text{m}$  in thickness) were obtained from frozen cecum samples, mounted on polylysine-coated slides, and fixed in acetone. They were incubated overnight with DPBS containing 10% horse serum to block nonspecific binding sites, and after incubation were washed and then incubated with primary antibodies (mouse anti-chicken CD4 and mouse anti-chicken CD8 known to cross react with turkey CD4 and CD8 respectively). They were then washed to remove any unbound primary antibody and incubated with a secondary antibody (biotinylated horse anti-mouse IgG). The sections were washed once more and then incubated with avidin-biotin peroxidase complex followed by 3, 3'-diaminobenzidine and counterstained with Harris Hematoxylin. The percentage area occupied by immunostained CD4 and CD8 lymphocytes in each section was estimated using Image-Pro Plus 6.2 (Media Cybernetics, Silver Springs, MD) connected to an Olympus BX50 light microscope (Olympus, Center Valley, PA) at 100 x magnification.

***Isolation of RNA, Reverse Transcription, and Real-Time PCR.*** Total RNA was isolated from samples in RNeasy<sup>®</sup> and reverse transcription to cDNA was carried out as described by Gadde et al. (2011). Differential expression of CXCLi2, interleukin (IL) 1 $\beta$ , interferon (IFN)  $\gamma$ , IL10, IL13, IL2, IL12b and IL18 was assessed by real-time PCR using an Applied Biosystems 7300 sequence detection system (Applied Biosystems). Primer and probe sequences used for CXCLi2, IL1 $\beta$ , IFN $\gamma$ , IL10 and IL13 were those presented in Gadde et al. (2011). For IL2, IL12b and IL18 primers and probe sequences (Table 1) were designed by PrimerQuest<sup>SM</sup> software (Integrated DNA Technologies Inc., Coralville, IA). A pool of cDNA prepared from ceca of uninfected controls on d 0 was used as the calibrator sample. All the samples were analyzed in triplicate for target genes and in duplicate for endogenous control (28s RNA).

Differences in the relative expression of target genes (mean fold changes in comparison to a calibrator sample) were quantified by delta delta C<sub>T</sub> method.

### ***Data analysis***

All the data were expressed as mean  $\pm$  SEM and analyzed using SAS<sup>®</sup> Enterprise Guide 4.2<sup>®</sup> (SAS Institute Inc, 2009). Statistical analysis of weight gain data was carried out by one-way ANOVA and the means were separated using Student-Newman-Keuls test. The data for leukocyte infiltration, percentages of CD4<sup>+</sup>, CD8<sup>+</sup> lymphocytes and relative gene expression were analyzed by Student's t-test. Results with p-value  $\leq 0.05$  were considered as significantly different.

## **RESULTS**

### ***Oocyst excretion***

No oocysts were produced in the feces of uninfected birds (data not given). The number of oocysts produced in the feces of infected birds is shown in Fig.1. Three peaks in oocyst production were observed when birds were 7, 13, and 18 d of age; each peak was followed by a decline. The peak on d 7 was significantly smaller than those on d 13 and 18 and occurred 7 d after the inoculation of 10<sup>2</sup> oocysts on d 0. The peaks on d 13 and 18 occurred 7 and 6 d after inoculation of birds with 10<sup>3</sup> and 10<sup>4</sup> oocysts on d 6 and 12 respectively.

### ***Acquisition of immunity***

**Weight gain.** Results are presented in Table 2. The weight gain of IC birds was not significantly different from UC birds when challenged on d 6 with  $5 \times 10^4$  oocysts indicating that protection had not been acquired against *E. adenoeides* as a result of infection on d 0 with  $10^2$  oocysts. Weight gain of IC birds was significantly greater than UC birds but less than the UU controls when challenged on d 12 indicating that partial protection had been acquired following infection with  $10^2$  and  $10^3$  oocysts on d 0 and 6. Weight gain of IC birds was significantly greater than UC birds but less than the UU controls when challenged on d 18 indicating that complete protection against challenge had not been obtained following infection with  $10^2$ ,  $10^3$ , and  $10^4$  oocysts on d 0, 6, and 12 respectively.

**Mortality.** Mortality of IC birds was similar to UC birds when challenged on d 6 with  $5 \times 10^4$  oocysts indicating that no protection had been acquired against *E. adenoeides*. No mortality occurred in IC poultts when challenged on d 12 or d 18 indicating that protection had been acquired following exposure to  $10^2$  and  $10^3$  oocysts or  $10^2$ ,  $10^3$ , and  $10^4$  oocysts respectively.

### ***Cellular immune responses***

**Leukocyte infiltration and Immunohistochemistry.** Cecal sections analyzed for leukocyte infiltration showed significantly higher scores in infected poultts compared to sections from uninfected poultts on d 6, 10, 12, 16 and 18 (Fig. 2). The infiltrating cells were a mixture of granulocytes and mononuclear cells during the early stages of infection (d 4, 6, 10) and were dominated by mononuclear cells in the later stages of infection (d 12, 16, 18). Sections analyzed

by immunohistochemistry showed significantly higher levels (% Area) of CD4+ and CD8+ lymphocytes in infected poult compared to uninfected controls on d 12, 16 and 18 (Fig. 3).

**Real-time PCR.** Mean fold changes of chemokines and cytokines in both infected and uninfected poult were shown in Fig. 4. The expression of CXCLi2 and IL10 was elevated on d 12, IL13 & IL18 on d 10, IL2 on d 10, 16 and that of IL12b on d 16 compared to uninfected poult ( $P < 0.05$ ). IFN $\gamma$  expression was found to be higher in infected poult on d 10, 12, and 16 compared to uninfected controls. No significant changes ( $P < 0.05$ ) were found in the mean fold changes of IL1 $\beta$  between infected and control poult.

## DISCUSSION

Little is known regarding the acquisition of protective immunity and cellular immune responses to infection when turkey poult are infected with oocysts of *Eimeria* early in life. In this experiment, poult were initially infected with  $10^2$  oocysts following transfer from the hatchery, such exposure to a low dose of oocysts could occur following placement of commercial poult upon litter. Poult were reinfected with  $10^3$  and  $10^4$  oocysts at 6 and 12 d of age in order to simulate secondary exposure to infection that in chickens has been shown to be necessary for the development of immunity (Chapman et al., 2005).

Oocysts produced in the feces of poult given this dosing regimen indicated that an initial small peak in numbers occurred 7 d after inoculation of  $10^2$  oocysts. This was followed by two larger peaks 7 and 6 d after inoculation of  $10^3$  and  $10^4$  oocysts respectively. The prepatent period of *E. adenoides* (time from inoculation of oocysts to the first appearance of new oocysts in the feces) was reported by Clarkson (1958) to be 114 - 132 hr. According to Hein (1969), maximum

numbers of new oocysts occur from 6-7 d following infection with *E. adenoeides*, a finding similar to that found in the present study. The presence of a third peak in oocyst production at 18 d of age indicates that the parasite is still capable of multiplying in the host following infection with increasing doses of oocysts given on three successive occasions.

The results indicate that judged by weight gain no protection had been acquired when poult were challenged at 6 d of age but poult challenged at 12 and 18 d had acquired partial protection. Judged by mortality, however, complete protection against challenge had been acquired by d 12 and d 18. The results of the present study suggest that secondary exposure of poult to oocysts in the litter is important in order for protective immunity to develop against *E. adenoeides*. In another study, protection at 3 wk of age was demonstrated following inoculation of poult at 1, 7, and 14 d of age (Augustine, 1988). However a much larger dose of oocysts ( $5 \times 10^4$  / poult) was employed and the dose comprised a mixture of two species (*E. adenoeides* and *E. meleagrimitis*) so that it was not possible to conclude which was responsible for protection.

Nothing is known regarding cellular immune responses when poult are infected with a series of doses at an early age. Such responses were characterized by measuring the leukocyte infiltration, percentage area occupied by CD4+ and CD8+ lymphocytes and the relative expression of various cytokines in the ceca. A progressive increase in leukocyte infiltration score was seen in sections of ceca from infected poult from d 6-16 compared to uninfected controls. The majority of the infiltrating cells were mononuclear phagocytes (macrophages and lymphocytes), but moderate numbers of granulocytes (heterophils, eosinophils, basophils) were also seen during early stages of the infection. This significant increase in the number of infiltrating cells indicates the inflammatory and innate immune activities occurring in response to infection.

Adaptive immune mechanisms can involve cell mediated and antibody mediated responses, the development of which is directed by T helper-type 1 (Th1) cells and Th2 cells, respectively. *Eimeria* infections are known to induce both Th1- and Th2-mediated responses, although Th1-activities play a predominant role (Lillehoj and Trout, 1996). Characterization of the subsets and evaluation of the number of immune cells, mainly T lymphocytes, is important in understanding immune activity occurring in response to infection. In this study, an increase in the percent area occupied by specific subsets of T-lymphocytes (CD4+ and CD8+) was seen in the ceca of infected poult indicating the development of adaptive immune activities in response to infection. The increase in CD4+ and CD8+ lymphocytes on d 12, 16, and 18 is in agreement with the observed mononuclear cell infiltration at these time points and with the expected time-course of lymphocyte infiltration during the effector-phase of an adaptive cell-mediated immune response.

The relative expression of various cytokine and chemokine levels was found to be altered in response to infection. Chemokines are a group of small, structurally related cytokines that show chemotactic activity to leukocytes (Kaiser et al., 1999; Sick et al., 2000). They are grouped into four major families (CXC, CC, XC, CX3C) based upon the arrangement of cysteine residues in their amino terminal end (Zlotnik and Yoshie, 2000). More than 20 types of chemokines have been identified from chickens (DeVries et al., 2006). CXCLi2 is an important avian pro-inflammatory chemokine, reported to have structural and functional similarities with human IL8 (Kaiser et al., 1999; Mayne et al., 2007). Its major role is to attract lymphocytes and monocytes to the site of infection (Laing and Secombes, 2004). In the current study, the relative expression level of chemokine CXCLi2 was found to be significantly elevated on d 12 in infected poult, though increased levels were also seen on d 6 post-infection. The increase in the

relative expression of CXCLi2 suggests a role for this chemokine in attracting leukocytes to the site of infection.

IL1 $\beta$  is a pro-inflammatory cytokine produced by activated mononuclear phagocytes, neutrophils, epithelial cells, and endothelial cells (Abbas et al., 2010) that plays an important role in recruitment of phagocytic cells to site of infection, induction of T cell proliferation and B cell maturation, activation of cytokine production, and induction of release of chemokines (Weining et al., 1998). In this study, no significant changes between infected and uninfected poult were found in the levels of IL1 $\beta$ , but increased levels were found in infected poult on d 6. The increased numbers of macrophages and heterophils seen to infiltrate the ceca during early stages of infection could be the source of the elevated levels of IL1 $\beta$  observed in this study and might stimulate the recruitment of more leukocytes into the ceca.

IFN $\gamma$  is an important mediator of resistance to coccidiosis in chickens (Rose, 1982) that is produced mainly by Th1 lymphocytes, activated cytotoxic T cells, and natural killer (NK) cells. Its production can be used as a measure of T cell responses to coccidial antigens (Prowse and Pallister, 1989). In this study, IFN $\gamma$  levels were found to be significantly increased on d 10, 12 and 16 in infected poult following infection compared to controls. These increased levels might be a reflection of the effector function of recruited CD4 $^{+}$  and CD8 $^{+}$  lymphocytes shown to be present in the cecum on d 12, 16 and 18.

Interleukin 10 is known to regulate immune responses in *Eimeria* by inhibiting the production of pro-inflammatory cytokines, MHC expression, and nitric oxide production, by direct effects upon T cells, B cells, NK cells, and antigen presenting cells (Rothwell et al., 2004). Major sources of IL-10 include activated T cells, B cells, macrophages, mast cells, and epithelial cells. In the current study, IL10 expression was found to be significantly elevated on d 12 in

infected poult in comparison to uninfected poult. While generally considered an anti-inflammatory cytokine, IL10 has been reported to have immunostimulatory effects in chickens by increasing IFN $\gamma$  production, and supporting NK and cytotoxic T cell activity in mice, humans and chickens (Shibata et al., 1998; Lauw et al., 2000; Abdul-Careem et al., 2008, Shi and Erf, 2012). The high expression of IL10 at a time (d 12) when expression of IFN $\gamma$  is increasing and continues to be elevated (d 16), suggests a positive effect of IL10 in the progression of cell-mediated immune activities during *Eimeria* infection in poult.

IL13 is a cytokine produced mainly by Th2 cells, CD8 $^{+}$  T cells, NK cells, basophils eosinophils and epithelial cells that, in addition to its major role in isotype switching by B cells, also promotes inflammation by recruitment of granulocytes and monocytes into tissues (Abbas et al., 2010). Significantly increased expression of IL13 was seen on d 10 in infected poult compared to uninfected controls. IL13 during this time of infection may be produced by local intraepithelial lymphocytes, eosinophils and basophils in the ceca. Absence of elevated levels of IL13 in the later stages of infection (d 12, 16 and 18), when there is an increase in CD4 $^{+}$  and CD8 $^{+}$  lymphocytes in the ceca, indicates the development of a Th1 response rather than a Th2 response (Gadde et al., 2011).

IL2 plays an important role as a growth, survival and differentiation factor for antigen-activated T cells, NK cells, and  $\gamma\delta$  T cells, it stimulates cytokine production by T-helper and NK cells, and also is required for survival and function of regulatory T cells (Lawson et al., 2000; Abbas et al., 2010). The increased expression levels of IL2 on d 10 and d 16 in infected poult could be correlated with the increased levels of CD4 $^{+}$  and CD8 $^{+}$  lymphocytes from d 12, suggesting a role for IL2 as a promoter of T cell growth and survival in *E. adenoeides* infections. Cornelissen et al. (2009) reported a similar increase in IL2 expression in the duodenum and



cecum of chickens infected with *E. acervulina* and *E. tenella*, respectively and Choi and Lillehoj (2000) observed an increase in IL2 mRNA expression in the spleen and duodenum of chickens infected with *E. acervulina*.

IL12b is the beta subunit of cytokine IL12, produced mainly by activated dendritic cells and macrophages. The major functions of IL12 are stimulation of production of IFN $\gamma$  by T lymphocytes and NK cells, synergistic action with IFN $\gamma$  to promote the differentiation of CD4+ helper T lymphocytes, and enhancement of the cytotoxic function of CD8+ T cells and NK cells (Abbas et al., 2010). In the present study, increased levels of IL12b were found in infected poult starting from d 10, but a significant increase in the levels was seen only on d 16 compared to uninfected poult. These observations appear to correlate with the increased expression of IFN $\gamma$  on d 10, 12 and 16, supporting the role of IL12 as stimulator of IFN $\gamma$  production. IL12 can also stimulate CD4+ and CD8+ T lymphocytes to produce IL10 (Gerosa et al., 1996; Abdul-Careem et al., 2008), which could explain the simultaneous up-regulation of IFN $\gamma$  and IL10 on d 12 observed in the present study.

IL18 also acts synergistically with IL12 in enhancing IFN $\gamma$  production by T cells and promotes differentiation of CD4+ T cells (Kaiser, 2002; Abbas et al., 2010). Dendritic cells and macrophages are the major sources of IL18 production. In this study, IL18 was found to be significantly elevated on d 10 following infection. The increase in IL18 expression along with IL12b could contribute to the increase in IFN $\gamma$  production. Acting together, IL12b, IL18, and IFN $\gamma$  may act as inducers of cell-mediated immunity in response to infection with *E. adenoeides*.

In conclusion, the results of this study characterize the acquisition of immunity and the development of cell-mediated immune activities in response to a progressive *E. adenoeides* infection in turkey poult.

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**Table 1.** Primer and probe sequences used to assay turkey cytokines IL2, IL12b and IL18 by Real Time PCR

Target	Primer / probe sequence	Accession no.
IL2	Turkey	AJ007463
Forward primer	5'-CAGATAATCGGGACACTGAC-3'	
Reverse primer	5'-CGATGCTCCATAAGCAGTAG-3'	
Probe	5'-CTTCAGCTGTATTTTCAGTAGCACTGC-3'	
IL12b	Turkey	AJ564203
Forward primer	5'-CCCACCTCAATGTCAGTATG-3'	
Reverse primer	5'-GGAAGTAGGACTTTGGTGTG-3'	
Probe	5'-CACAAATGGAACAGTGACCTGGAC-3'	
IL18	Turkey	AJ312000
Forward primer	5'-GATCAGGAGGTGAAATCTGG-3'	
Reverse primer	5'-CTGAAGGTGCAGTAGTCTTG-3'	
Probe	5'-GTGGAATGTACTTCGACATTCACTG-3'	

<sup>a</sup>IL = Interleukin

<sup>b</sup>All probes were labeled with 6-carboxyfluorescein at the 5' terminus and 6-carboxytetramethylrhodamine at the 3' terminus.

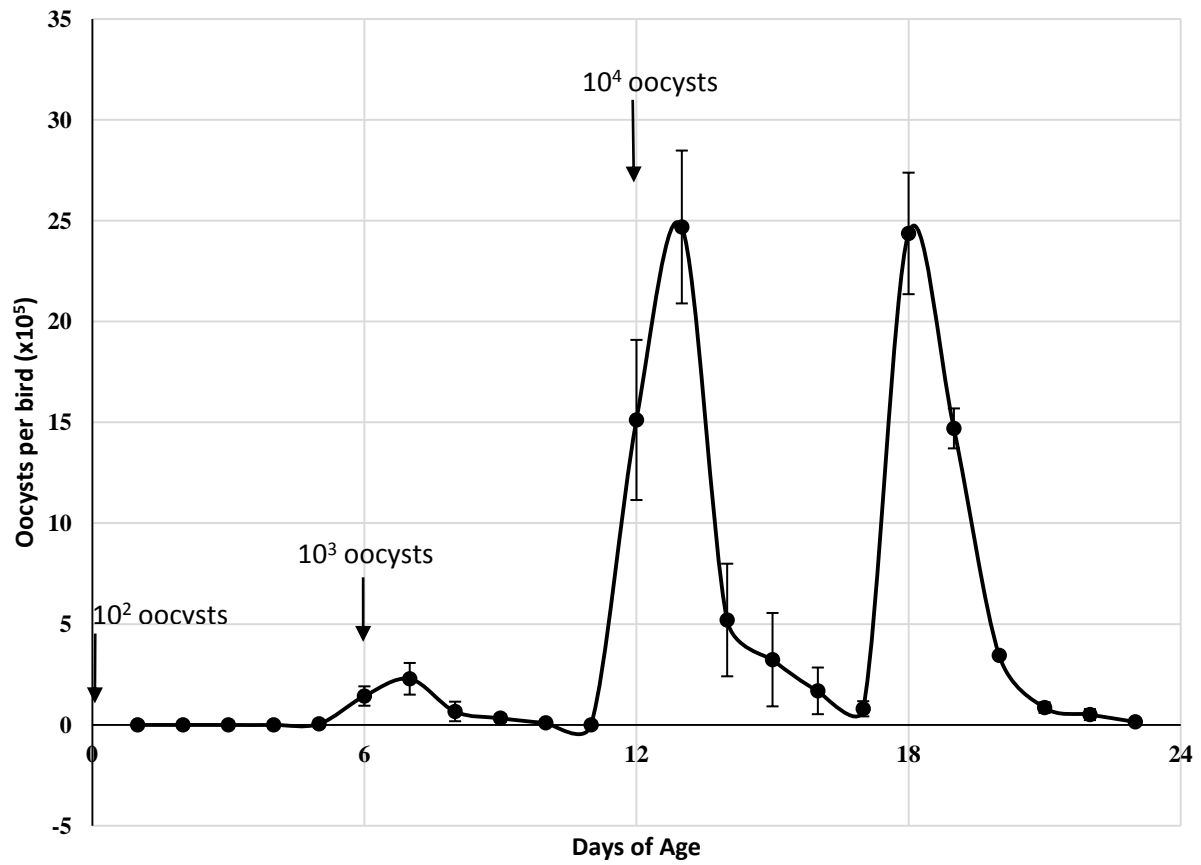
**Table 2.** Mortality and bodyweight gain of poult<sup>1</sup> challenged with  $5 \times 10^4$  oocysts of *E. adenoeides* at 6, 12, and 18 d of age

Treatment <sup>2</sup>	Day of challenge					
	6		12		18	
	Weight gain (g)	% mortality	Weight gain (g)	% mortality	Weight gain (g)	% mortality
IC	$37 \pm 6^b$	58.3	$119 \pm 10^b$	0	$228 \pm 6^b$	0
UC	$32 \pm 2^b$	87.5	$46 \pm 3^c$	83.3	$67 \pm 11^c$	37.5
UU	$129 \pm 5^a$	0	$207 \pm 5^a$	0	$276 \pm 4^a$	0

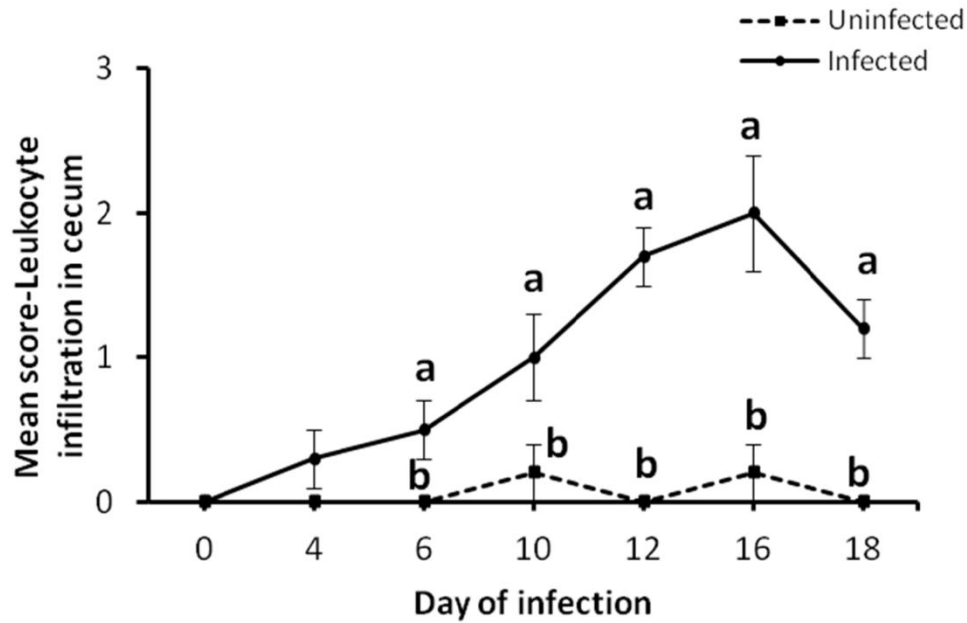
Values in a column with no common superscript differ significantly ( $P \leq 0.05$ ).

<sup>1</sup> Poult<sup>s</sup> were infected with  $10^2$  oocysts at placement and reinfected with  $10^3$  and  $10^4$  oocysts when 6 and 12 d of age respectively or given a sham dose of water.

<sup>2</sup>IC = infected challenged birds; UC = Uninfected challenged birds; UU = Uninfected unchallenged birds

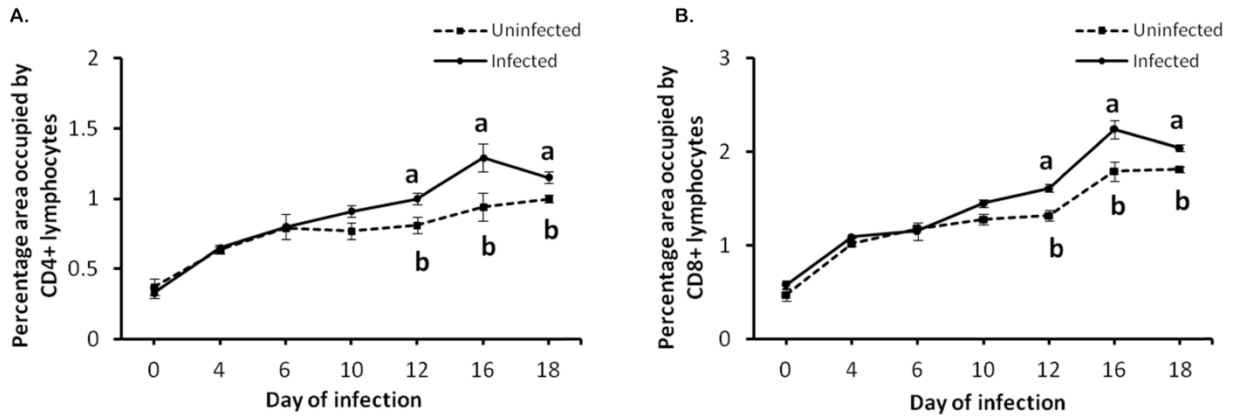


**Figure 1.** Oocysts produced in the feces of poults (Mean  $\pm$  SEM) following infection with  $10^2$  oocysts on d 0 and subsequent reinfection with  $10^3$  and  $10^4$  oocysts on d 6 and d 12 respectively.

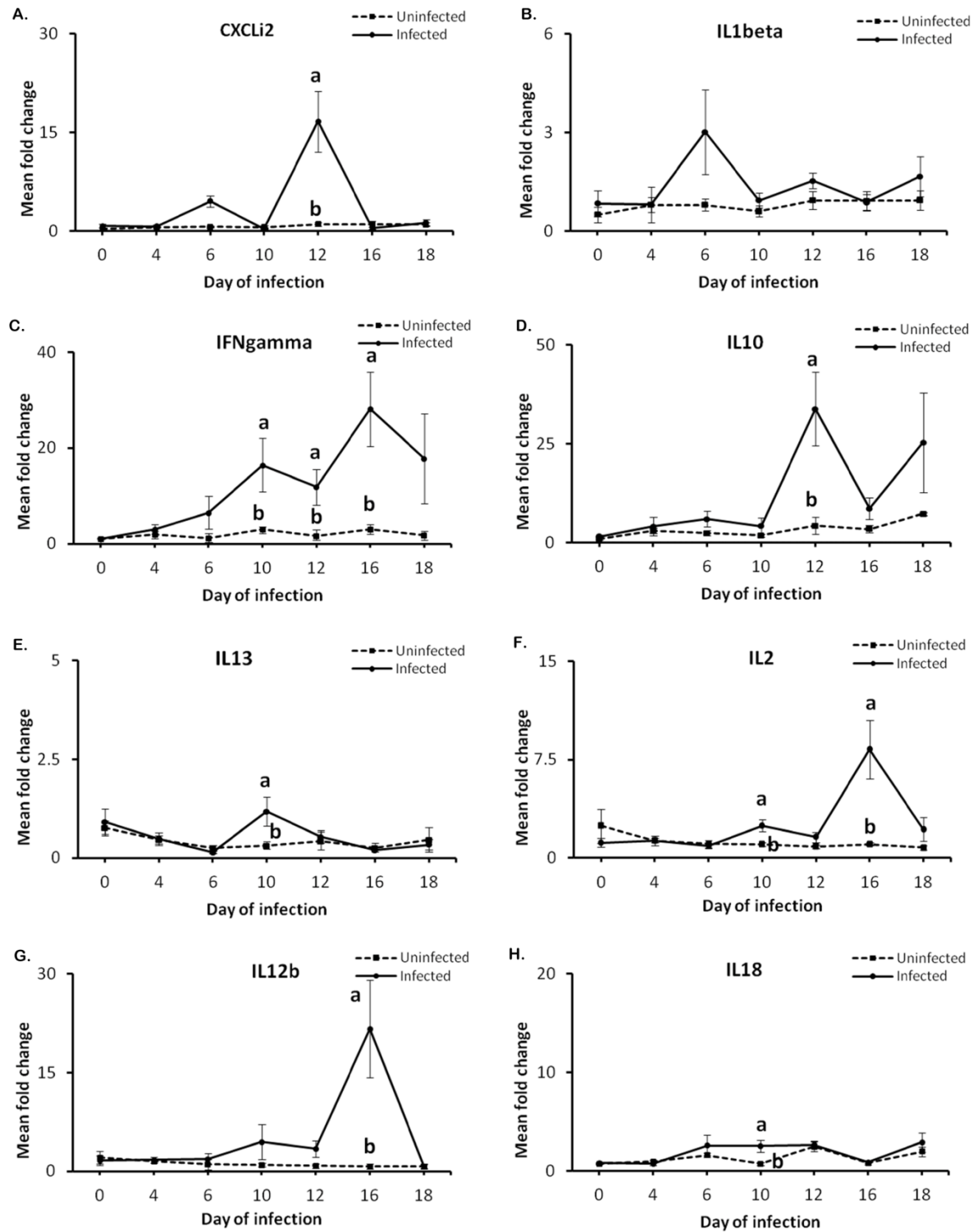


**Figure 2.** Severity of leukocyte infiltration in sections of ceca from turkey poult infected with  $10^2$  oocysts / poult on d 0 and subsequently re-infected with  $10^3$ ,  $10^4$  oocysts / poult on d 6 and 12 of age respectively or given sham dose of water. Sections were stained with hematoxylin & eosin and observed under 100X objective. They were given scores ranging from 0-3 based on the extent and severity of infiltration. Results were expressed as mean  $\pm$  SEM for each d of infection. Values with no common letter differ significantly ( $P \leq 0.05$ ).





**Figure 3.** Percentage area occupied by A) CD4+, and B) CD8+ lymphocytes in immunohistochemically stained sections of ceca analyzed with Image-Pro Plus Software. Samples were collected from poult infected with  $10^2$  oocysts / poult on d 0 and subsequently re-infected with  $10^3$ ,  $10^4$  oocysts / poult on d 6 and d 12 of age respectively or given sham dose of water. Results were expressed as mean  $\pm$  SEM. For each d of infection, values with no common letter differ significantly ( $P \leq 0.05$ ).



**Figure 4.** Mean fold change in the relative expression of chemokine [A) CXCLi2] and various cytokines [B) IL1 $\beta$ , C) IFN $\gamma$ , D) IL10, E) IL13, F) IL2, G) IL12b, and H) IL18] following

infection of newly hatched turkey poults with a series of doses of *Eimeria adenoeides*. Relative gene expression was measured by real-time, reverse-transcription PCR. The data were analyzed using comparative C<sub>T</sub> method and expressed as fold change in comparison to a calibrator sample. A pool of cDNA from ceca of uninfected controls on d 0 was used as a calibrator sample.

<sup>a,b</sup> For each d of infection, treatment means with no common letter differ significantly ( $P \leq 0.05$ ).

For each treatment, values are the mean  $\pm$  SEM for six poults.



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## MEMORANDUM

TO: H. D. Chapman  
FROM: Craig N. Coon, Chairman  
Institutional Animal Care  
And Use Committee  
DATE: October 15, 2009  
SUBJECT: IACUC PROTOCOL APPROVAL  
Expiration date : October 31, 2012

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol #10011-  
“**DEVELOPMENT OF IMMUNITY TO EIMERIA ADENOEIDES IN THE TURKEY**”. You may  
begin this study immediately.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any  
changes in the protocol during the research, please notify the IACUC in writing **prior** to initiating the  
changes. If the study period is expected to extend beyond **10-31-2012**, you must submit a new protocol.  
By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for  
research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian



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Attn University of Arkansas Graduate School

June 20, 2013

Dear Sir,

I attest that Ujvala Deepthi Gadde was first author of the manuscript cited below and completed at least 51% of the work for the paper.

Gadde, U., T. Rathinam, G. F. Erf, and H. D. Chapman. Acquisition of Immunity to the Protozoan Parasite *Eimeria adenoeides* in Turkey Poult and Cellular Responses to Infection (Poult. Sci.)-Submitted for publication

Yours Sincerely,

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## CONCLUSION

Coccidiosis is a common enteric disease of turkeys and is caused by protozoan parasites belonging to the genus *Eimeria*. Infection with *Eimeria* was known to induce long lasting protective immunity in chickens, and extensive research was conducted on the role of various immune cells involved in development of immunity. In the turkey, acquisition of immunity plays an important role in the control of the disease and yet almost nothing is known regarding the mechanisms of immunity in this host.

In experiment 1 (Chapter II), we showed that judged by weight gain and oocyst production, poults had acquired immunity to *E. adenoeides* as a consequence of exposure to  $12.5 \times 10^3$  oocysts. The alterations in the concentrations of WBC populations observed during primary infection with *E. adenoeides* are supportive of initiation of cellular immune activities involving components of both innate and adaptive immunity. The increase in peripheral blood lymphocytes and monocytes on d 7 together with the increased CD4<sup>+</sup> to CD8<sup>+</sup> lymphocyte ratios on day 4 and 11, and the increase in CD8<sup>+</sup> lymphocytes on d 11 collectively point towards initiation of cell-mediated immunity and the changes in blood does reflect the mobilization and recruitment of cells in response to infection. It is concluded that cell-mediated immunity is responsible for the observed protection in the challenge study. Taken together, based on changes in WBC populations during the course of a primary infection with *E. adenoeides* in turkey poults, effector immune mechanisms designed to deal with different stages of the parasites have been initiated (extracellular stages: eosinophils; intracellular stages: CD8<sup>+</sup> lymphocytes and monocytes / macrophages). While emphasis is on cellular immune activities, both CD4<sup>+</sup> T helper cell type 1 (Th1) and CD4<sup>+</sup> Th2 lymphocytes are likely involved in the coordination of these protective efforts.

Experiment 2 (Chapter III) showed that infiltration with mononuclear leukocytes was significantly elevated in infected poult and remained high throughout the course of infection indicating the initiation of immune activities in the host. An increase in the area occupied by T helper and cytotoxic lymphocytes suggests greater numbers of these cells at the site of parasite multiplication and indicates initiation of specific effector cell mediated immune activities against parasitic stages in intestinal tissue. While specific subsets of T cells were found elevated in stained sections only from d 9, increased infiltration of mononuclear leukocytes was observed from d 4 following infection. The leukocytes infiltrating cecal tissue on d 4 and d 7 may be mostly macrophages, along with CD4- and CD8-negative lymphocytes such as  $\gamma\delta$  T cells and B cells that were recruited to the mucosa due to the inflammatory activities initiated by infection. On the other hand, the infiltration of CD4+ and CD8+ lymphocytes observed on d 9 and 11 post-infection may mark the arrival of effector lymphocytes (T helper and cytotoxic, respectively). The cytokine expression profiles during the early phase of *Eimeria* infection may be attributed to inflammatory activities of local macrophages, injured tissue cells, mast cells, NK cells, IEL and epithelial cells as well as recruited macrophages and lymphocytes. The arrival of CD4+ and CD8+ lymphocytes on d 9 and 11, together with a variable increase in IFN $\gamma$  expression, appears to mark the adaptive phase of the response to *Eimeria* infection in the cecum.

In experiment 3 (Chapter IV), we showed that judged by weight gain no protection had been acquired when poult were challenged at 6 d of age but poult challenged at 12 and 18 d had acquired partial protection. Judged by mortality, however, complete protection against challenge had been acquired by d 12 and d 18. The results suggest that secondary exposure of



poults to oocysts in the litter is important in order for protective immunity to develop against *E. adenoeides*.

In conclusion, this study has successfully demonstrated the acquisition of immunity, changes in leukocyte infiltration, percent area occupied by subsets of lymphocytes, and expression of various chemokines and cytokines in response to *E. adenoeides* infection. This study lays a solid foundation for further studies into the immunological aspects underlying the development of resistance and immunity to *E. adenoeides*.